LFA-1 Integrin β Chain Phosphorylation Regulates Protein Interactions and Mediates Signals in T Cells

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SUMMARY OF THE STUDY

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-V:

I. Fagerholm, S.C.*, Hilden, T.J.*, Nurmi, S.M. and Gahmberg, C.G. (2005) Specific integrin α and β chain phosphorylation regulate LFA-1 activation through affinity-dependent and -independent mechanisms. *J. Cell Biol.* **171**, 705-15

II. Nurmi, S.M., Gahmberg, C.G. and Fagerholm, S.C. (2006) 14-3-3 proteins bind both filamin and alphaLbeta2 integrin in activated T cells. *Ann N Y Acad Sci.* **1090**

III. Nurmi, S.M., Autero, M., Raunio, A.K., Gahmberg C.G. and Fagerholm, S.C. (2007) Phosphorylation of the LFA-1 integrin β_2-chain on Thr-758 leads to adhesion, Rac-1/Cdc42 activation, and stimulation of CD69 in human T cells. *J Biol Chem.* **282**, 968-75

IV. Choi, E.Y., Orlova, V.V., Fagerholm, S.C., Nurmi, S.M., Zhang, L., Ballantyne, C.M., Gahmberg, C.G. and Chavakis, T. (2008) Regulation of LFA-1-dependent inflammatory cell recruitment by Cbl-b and 14-3-3 proteins. *Blood.* **111**, 3607-14

V. Takala, H.*, Nurminen, E.*, Nurmi, S.M.*, Aatonen, M., Strandin, T., Takatalo, M., Kiema, T., Gahmberg, C.G., Ylänne, J., Fagerholm, S.C. (2008) Integrin β2 phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. *Blood.* **112***,* 1853-62

*These authors have contributed equally to the work.

ABBREVIATIONS

ADAP	adhesion- and degranulation-promoting adaptor
ADMIDAS	adjacent to the MIDAS
AP-1	activator protein
APC	antigen presenting cell
BMDM	bone marrow-derived mononuclear phagocytes
DAG	diacylglyserol
ECM	extracellular matrix
Erk	extracellular signal-regulated kinases
FAK	focal adhesion kinase
FERM	band 4.1, ezrin, radixin, moesin
FRET	fluorescent resonance energy transfer
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
HEV	high endothelial venules
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetes mellitus
I-EGF	integrin-epidermal growth factor
IgFLNa21	filamin immunoglobulin-like domain 21
IP3	inositol-tris-phosphate
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
JAB-1	Jun-activation domain-binding protein-1
Jnk	c-Jun N-terminal kinase
LAD	leukocyte adhesion deficiency
LAT	linker for activation of T cells
LFA-1	leukocyte function-associated antigen-1
MAP kinase	mitogen-activated protein kinase
MHC	major histocompatibility complex
MIDAS	metal ion dependent adhesion site
MS	multiple sclerosis
PDBu	phorbol 12,13 dibutyrate
PH	plecstrin homology
PI3-kinase	phosphoinisitide 3-OH kinase
PIP3	phosphatidylinositol triphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PTB	phosphotyrosine-binding
PtdIns(4,5)P2	phosphaditylinositol (4,5)-biphosphate
Rack1	receptor for activated C-kinase 1
RapL	regulator for cell adhesion and polarization
RGD	Arg-Gly-Asp peptide
RIAM	Rap1-guanosine triphosphate-interacting adaptor
SKAP55	Src kinase-associated phosphoprotein of 55 kD
SLP76	SH2 domain-containing leukocyte phosphoprotein of 76 kD
TCR	T cell receptor
TM	transmembrane

ABSTRACT

Integrins are heterodimeric transmembrane adhesion receptors composed of α- and β-subunits and they are vital for the function of multicellular organisms. Integrin-mediated adhesion is a complex process involving both affinity regulation and coupling to the actin cytoskeleton. Integrins also function as bidirectional signaling devices, regulating cell adhesion and migration after inside-out signaling, but also signal into the cell to regulate growth, differentiation and apoptosis after ligand binding. The LFA-1 integrin is exclusively expressed in leukocytes and is of fundamental importance for the function of the immune system.

The LFA-1 integrins have short intracellular tails, which are devoid of catalytic activity. These cytoplasmic domains are important for integrin regulation and both the α and β chain become phosphorylated. The α chain is constitutively phosphorylated, but the β chain becomes phosphorylated on serine and functionally important threonine residues only after cell activation. The cytoplasmic tails of LFA-1 bind to many cytoskeletal and signaling proteins regulating numerous cell functions. However, the molecular mechanisms behind these interactions have been poorly understood. Phosphorylation of the cytoplasmic tails of the LFA-1 integrin could provide a mechanism to regulate integrin-mediated cytoskeletal interactions and take part in T cell signaling.

In this study, the effects of phosphorylation of LFA-1 integrin cytoplasmic tails on different cellular functions were examined. Site-specific phosphorylation of both the α- and β-chains of the LFA-1 was shown to have a role in the regulation of the LFA-1 integrin. α-chain Ser1140 is needed for integrin conformational changes after chemokine- or integrin ligand-induced activation or after activation induced by active Rap1, whereas β-chain binds to 14-3-3 proteins through the phosphorylated Thr758 and mediates cytoskeletal reorganization. Thr758 phosphorylation also acts as a molecular switch to inhibit filamin binding and allows 14-3-3 protein binding to integrin cytoplasmic domain, and it was also shown to lead to T cell adhesion, Rac-1/Cdc42 activation and expression of the T cell activation marker CD69, indicating a signaling function for Thr758 phosphorylation in T cells. Thus, phosphorylation of the cytoplasmic tails of LFA-1 plays an important role in different functions of the LFA-1 integrin in T cells.

It is of vital importance to study the mechanisms and components of integrin regulation since leukocyte adhesion is involved in many functions of the immune system and defects in the regulation of LFA-1 contributes to auto-immune diseases and fundamental defects in the immune system.

REVIEW OF THE LITERATURE

1. INTRODUCTION TO LEUKOCYTE INTEGRINS

Leukocytes or white blood cells are bone marrow-derived cells and principal components of the immune system. Leukocytes present in the blood respond to signals of tissue injury by adhering to vascular endothelial cells, then transmigrating across the endothelium through the basement membrane and homing to sites of infection or inflammation. In order for the leukocytes to adhere to other cells, they first need to become activated. Integrins play a key role in the control of leukocyte activation in both innate and adaptive immunity. Integrins form a superfamily of noncovalently linked heterodimeric cell surface receptors composed of α- and β-subunits. 18 α and 8 β subunits have been identified in humans forming 24 distinct heterodimers, which all appear to have a specific function. They all bind to specific extracellular matrix (ECM), cell-surface or soluble ligands (**Fig 1**). Both the α and β subunit are type I transmembrane glycoproteins that have a large extracellular domain and short cytoplasmic tail (β4 being an exception) (reviewed by Hynes, 2002). A specific integrin can be depicted by using the Greek symbols or the CD numbers as well as the name of the integrin. Leukocytes can express at least 12 of the 24 known integrin heterodimers, the expression pattern depending on the subset and maturation state of the cell (von Adrian and Mackay, 2000). The four leukocyte-specific β2 integrins (αLβ2, αMβ2, αXβ2, αDβ2) are found on T cells, αLβ2 or LFA-1 (this name will be used through out the text) being the most abundant and widespread in expression. Leukocytes also express the two β7 integrins (α4β7 and αEβ7) and, in common with many other cell types, the ECM-binding β1 integrins (α1-α6β1). LFA-1 is involved in a) the interaction between T-cells and antigen presenting cells, b) the adhesion of cells to post-capillary high endothelial venules or to activated endothelium at sites of inflammation (extravasation), c) the control of cell differentiation and proliferation, and d) the regulation of T-cell effector functions. Therefore, a precise understanding of the spatial and temporal control of LFA-1 interaction with its cellular counter-receptors, the intercellular adhesion molecules (ICAMs), in the various contexts, is of vital importance.

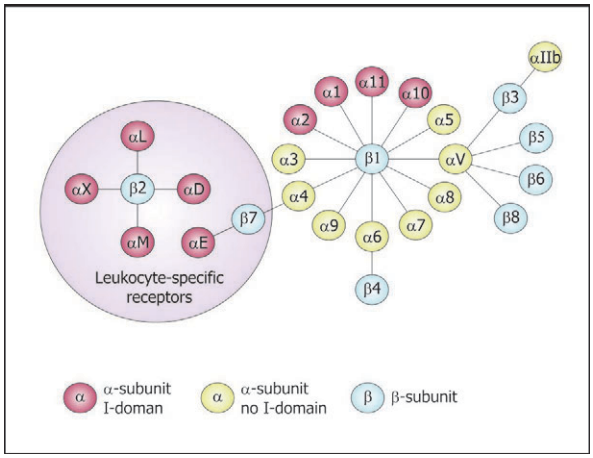

Fig 1. The members of the human integrin superfamily.
At least 18 α subunits and 8 β subunits have been identified, which are able to generate 24 different integrins. The 9 α subunits that contain the inserted I domain are shown in red and the α domains lacking the I domain in light green. The β subunits are shown in light blue. Leukocyte specific integrins are within the purple circle. Figure modified from Hynes, 2002.

Unstimulated lymphocytes are non-adherent, and in response to encountering chemokines or antigen, they become adherent to other cells and ECM components. Fundamental to this process is the ability of integrins to alter their avidity through an intracellular-signaling process that is known as inside-

out signaling (**Table 1**). This pathway ultimately modulates the affinity of integrins for their ligands (affinity regulation) and/or the extent to which integrins diffuse and cluster on the cell surface (valency regulation). In addition to affinity and valency regulation, interactions with and reorganization of the actin cytoskeleton contributes to the control of integrin-mediated adhesion. The triggering of inside-out signaling by chemokines or TCR leads to changes in cell morphology and to an increase in the avidity of integrins, which results in firm attachment to their ligands. This is followed by lymphocyte migration through the endothelium into lymphoid or inflamed tissues or to the formation of the immunological synapse and further T cell activation, respectively. In addition to mediating adhesion, integrins also function as signaling receptors, influencing cellular morphology, growth, and differentiation of the integrin-expressing cell, through a process known as outside-in signaling.

A detailed understanding of the molecular and cellular mechanisms that regulate integrin expression and adhesion will provide insight into the mechanisms of immune-cell trafficking and into the potential to manipulate these processes in therapeutic settings.

Mode of regulation	Explanation
Avidity	Avidity is defined by affinity and valency regulation
Affinity regulation of avidity	The strength of one individual receptor to bind ligand (monovalent interaction)
Valency regulation of avidity	Diffusion and clustering of several receptors in the plasma membrane to allow multivalent interactions with ligand

Table 1. Affinity and valency regulation of receptor avidity.

2. LEUKOCYTE FUNCTION ASSOCIATED ANTIGEN-1

2.1 LFA-1 in T cell adhesion

LFA-1 in T cell migration

Naïve T lymphocytes continually recirculate by crossing the high endothelial venules (HEVs) into lymph nodes and further on into the lymphatic circulation after which they re-enter the blood stream (von Andrian and Mempel, 2003, Cyster, 2005). If contact is made with the APC (antigen presenting cell), an immune response is initiated. All leukocytes are capable of migration, and a successful immune response critically depends on their capacity to be motile (Dustin, 2004).

The initial encounter with the vasculature involves the capture and tethering of leukocytes by selectin receptors that has the effect of progressively decreasing the leukocyte velocity (**Fig 2A**). Selectins are constitutively active and are specialized to engage rapidly and with high tensile strength. These initiators of the adhesion cascade comprise of L-selectin (expressed on leukocytes) and P- and E-selectin (expressed on endothelial cells) that all bind to oligosaccharides related to sialyl-Lewis^x. Selectin-mediated bonds are not strong enough to arrest cells at the vessel wall, but the contacts result in a rolling motion of the cells that is slower than that of free-flowing cells (von Andrian and Mempel,

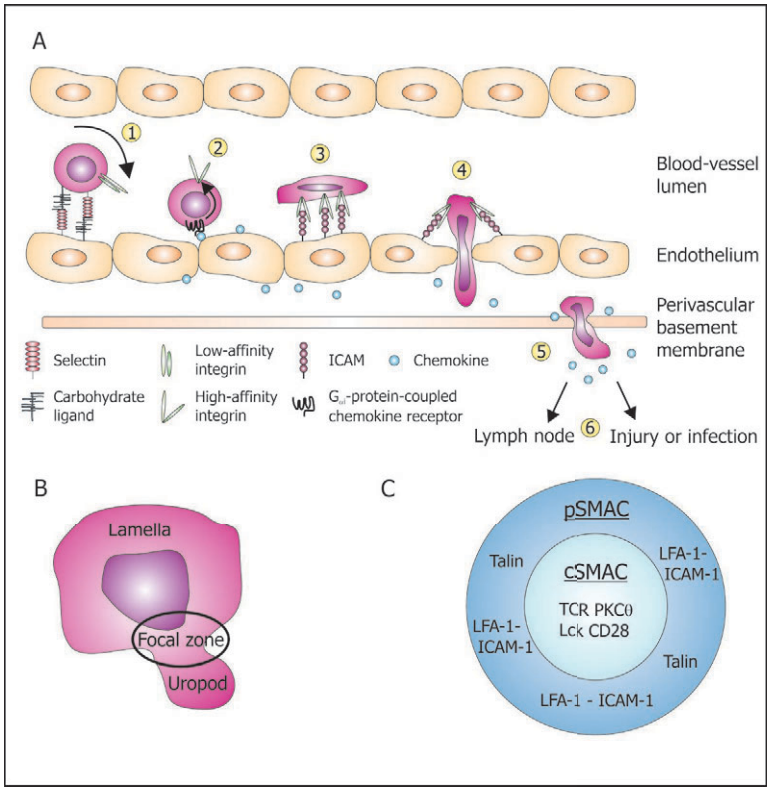

Fig 2. LFA-1 in T cell adhesion. **A. The multistep model of T cell migration.** Stages: 1. Rolling and tethering 2. Activation 3. Adhesion 4.Transendothelial migration 5. Migration across the PBM 6. Migration within the tissue. T cells migrate from the blood, across the vascular endothelium into infected tissue or into lymph nodes. Adapted from Kinashi, 2005. **B. Schematic picture showing the different zones in the migrating T cell.** The two active conformations of LFA-1 are differently distributed on the T cell membrane; high-affinity LFA-1 is located in a midcell 'focal zone' area and influences the speed of migration whereas intermediate affinity LFA-1 controls leading edge adhesions. Modified from Smith et al, 2007. **C. A stable immunological synapse viewed from the top.** cSMAC and the molecules found enriched in the central IS in light blue. LFA-1 and ICAM-1 are found along with talin in the pSMAC area of the IS (dark blue). Adapted form Friedl et al, 2005.

2003, Hogg et al, 2004, Smith et al, 2007). LFA-1 has been shown to contribute to the selectin-mediated leukocyte rolling by stabilizing the short-lived selectin bonds (Henderson et al, 2001). Once leukocytes have made contact with the blood vessel and have slowed down, they use LFA-1 to firmly attach. LFA-1 binds to intercellular adhesion receptors (ICAMs) which are expressed on chemokine activated endothelial cells (Berlin-Rufenach et al, 1999). Before actual cell migration, leukocytes undergo chemokine-induced polarization, with the formation of a projecting lamella at the leading edge, an elevated uropod at the trailing edge and a focal zone in the midcell area (**Fig 2B**). The distribution of LFA-1 in the migrating T-cell is somewhat surprising, since both the lamella and the midcell area express low levels of LFA-1 whereas the uropod expression level is high (Smith et al, 2005). However, the two active conformations of LFA-1 are also differently distributed on the T cell membrane of the migrating cell. By using antibodies that recognize different affinity conformations of LFA-1, it has been shown that LFA-1 in the leading edge is in the intermediate affinity conformation (Stanley et al, 2008). Also using the same technique it has been demonstrated that LFA-1 in the focal zone is in the high affinity conformation (Smith et al, 2005). The activation status of LFA-1 in the uropod is at present not known. However, neither of the antibodies described above, could bind LFA-1 in the uropod, which could indicate that the integrin is in a low-affinity conformation.

LFA-1 is also required for the next step of transmigration of naïve lymphocytes into lymph nodes or inflamed tissue (Berlin-Rufenach et al, 1999) and also for the recruitment of stimulated neutrofils into an inflammatory site (Henderson et al, 2003). ICAM-1 is expressed on the dorsal and the lateral surfaces of endothelial cells, and leukocytes transmigrate by following ICAM-1´s "tracks" (Oppenheimer-Marks et al, 1991). Lately new information has been presented on where exactly

leukocytes choose to transmigrate. They migrate preferentially through the endothelial junctions that display decreased levels of extracellular matrix molecules (Burns et al, 1997 and Wang et al, 2006). There is also increasing evidence that leukocytes may undergo transcellular migration, making use of LFA-1 through interaction with ICAM-1-expressing endothelial surfaces (Barreiro et al, 2002, Carman and Springer, 2004, Millan et al, 2006). However, the precise biological context of the two forms of transendothelial migration is yet to be determined, but these studies show that LFA-1 has an important role in all stages of the transmigration process.

In tissues T cells must not only cross the endothelial cell lining the vascular wall, but must also migrate through the perivascular basement membrane (PBM). Because the PBM is rich in integrin ligands, integrins are good candidates for mediating this process. Neutrophil migration across the endothelium has been shown to cause $\alpha 6\beta 1$ activation, which neutrophils use to migrate through the laminin-containing PBM (Dangerfield et al 2002). It is not known, however, whether the same applies to T cells. Once they have crossed the PBM, T cells follow the chemotactic gradient along large collagen fibres to the source of inflammation or injury (Dustin et al, 2004). When T cells are entering the lymph nodes, they enter via HEVs, and migrate to a specific destination at the edge of the node (paracortex). Selective responsiveness to chemokines is the driving force for this migration.

A very recent study has focused on the interplay between adhesive, contractile and protrusive forces during leukocyte migration (Lämmermann et al, 2008). These authors have studied how dendritic cells from mice lacking surface $\beta 1$, $\beta 2$ and $\beta 7$ (integrin$^{-/-}$ cells) migrate to T cell areas in the lymph node following the chemotactic gradient. Surprisingly, the integrin$^{-/-}$ cells and wild type cells both localized to the T cell area in an indistinguishable manner, suggesting that functional integrins do not contribute to migration in three-dimensions. However, the lymph node constitutes a fundamentally different environment for cellular migration, as it contains almost no freely accessible extracellular matrix molecules. Integrin-independent migration following the chemotactic gradient was also observed for B cells and granulocytes in artificial 3D matrices. When integrin$^{-/-}$ dendritic cells were tested on whether they could cross tissue barriers, neither endothelial nor epithelial lining was crossed, since these processes are integrin-dependent (Alon and Dustin, 2007). The cells are suggested to move in the three-dimensional environment by polarizing towards the chemotactic gradient. The leading edge and the trailing edge are functionally dissociated and the cells migrate by the sole force of actin-network expansion, which promotes protrusive flowing of the leading edge and contraction of the trailing edge. Thus, the authors suggest that the role of integrins is mostly to mediate retention, invasion, cell-cell communication and cell-cell adhesion (Lämmermann et al, 2008). However, whether this all applies to T cells, remains to be seen.

LFA-1 in the immunological synapse

To be able to transmigrate, T cells must interact with endothelium, but they are also able to interact with other cell types. Antigen presentation occurs when a T cell interacts with an antigen presenting cell (APC) that expresses its specific antigen bound to major histocompatibility complex (MHC) forming an MCH-peptide complex. When a T cells engages an APC, such as a dendritic cell, key receptors at the cell-cell contact reorganize to form the immunological synapse (IS) on the surface of the engaged T cell (Bromley et al, 2001, Cannon et al, 2002). The formation of the IS is a multistep process with two morphologically defined stages: the nascent or immature IS and the mature IS. In the nascent IS

LFA-1 is initially engaged in the centre of the contact area, with a peripheral ring of TCRs, but within minutes this distribution is reversed. In the mature IS the central area (central supramolecular activation cluster; cSMAC) becomes occupied by the TCRs, whereas LFA-1 becomes clustered as a peripheral ring (peripheral supramolecular activation cluster; pSMAC) colocalized with the cytoskeletal protein talin (Monks et al, 1998, Grakoui et al, 1999). Additional molecules involved in T cell activation are also clustered at the centre of the contact area, such as CD28, PKCθ, Lck, Fyn, CD4 and CD8 (Huppa et al, 2003) (**Fig 2C**). While stable IS formation may not be essential for T cell activation, this stable structure achieves the important goal of coordinating the antigen recognition process with migration, by stopping the T cell (Bromley et al, 2001, Cannon et al, 2002). The mature IS can be stable for more than 1h, however there is evidence of much more short-lived interactions (6-12 min) between T cells and dendritic cells, when T cells crawl over the surface of dendritic cells, seeking their specific MHC-peptide complex (Gunzer et al, 2000).

LFA-1 has also been shown to provide costimulatory signals that are required for full activation of signaling cascades (Sims and Dustin, 2002, Lin et al, 2005). Graf et al (2007) showed that coengagement of LFA-1 is not required for the formation of the cSMAC region, but does increase the accumulation of TCR/MHC complexes within the cSMAC. Moreover, LFA-1 is required for the recruitment and localization of talin into the pSMAC region and exclusion of CD45 from the synapse. Thus, in addition to its important role in regulating T cell-APC adhesion, coengagement of LFA-1 enhances T cell signaling, and this can be accomplished in part through the organization of proteins within the immunological synapse (Sims and Dustin, 2002).

2.2 LFA-1 in health and disease

Leukocyte adhesion deficiency and LFA-1 deficient mice

The physiological importance of β2 integrins in leukocyte function has been verified by the study of a naturally occurring human disease, leukocyte adhesion deficiency-I (LAD-I) (Hogg and Bates, 2000). LAD-I is a rare inherited immunodeficiency in which β2 integrin expression is diminished or lost. The majority of LAD-I cases involve single point 'missense' mutations in the β2 subunit gene giving rise to an altered precursor protein which then fails to bind to the α subunit precursor (Arnaout et al, 1993, Anderson et al, 1997). The absence or greatly reduced expression of β2 integrins results in elevated numbers of circulating neutrophils because the cells fail to migrate across the endothelium. Neutrophils rely most heavily on β2 integrins because they lack significant levels of other integrins. Patients typically have recurrent bacterial or fungal infections, impaired mobilization of leukocytes to infected areas, severe gingivitis, and impaired tissue remodelling and wound healing. Severely affected people often die of infections in early childhood unless bone marrow transplantation is successfully accomplished. A few variant LAD-I syndromes have also been identified (Kuijpers et al, 1997, Hogg et al, 1999 and McDowall et al, 2003). These variants exhibit rare mutations in the $\beta_2$integrin subunit CD18 with retained expression of this integrin subfamily but impaired functions. A novel LAD-I variant exhibits a major truncation in the β2 cytoplasmic domain, which impaired the binding function of LFA-1, but surprisingly not Mac-1 (αMβ2) (Hixson et al, 2004). This is the first report describing an LAD-I syndrome that specifically involves the cytoplasmic domain. Novel combined integrin activation deficiencies have recently emerged where leukocytes express intact integrins, but have impaired ability

to generate high avidity to endothelial ligands. These combined integrin activation deficiences are collectively termed LAD-III to avoid confusion with the LAD-I syndrome (Etzioni and Alon, 2007). An example of LAD-III deficiency is defective activation of Rap1 following chemokine activation, despite normal expression levels of Rap1, which leads to impaired stabilization of integrin bonds (Kinashi et al, 2004, Alon et al, 2003).

β2 deficient mice display a phenotype which is very similar to human LAD-I patients (Mizgerd et al, 1997, Scharffetter-Kochanek et al, 1998). The common features include leukocytosis, spontaneous skin infections, and impaired emigration of neutrophils at sites of infection. Furthermore, the impaired adherence of neutrophils and respiratory burst is similar in $\beta2^{-/-}$ mice and humans. β2 integrin is also crucial in regulatory T cell development and function (Marski et al, 2005). Mice with αL deficiency display a moderate defect in peritoneal emigration, reduced natural killer cytotoxicity and impaired rejection of tumour cells. However, they are still able to normally fight viral infection and they express normal levels of immunoglobulins (Schmits et al, 1996, Shier et al, 1996, 1999). Leukocytes deficient in αLβ2 display a severe impairment in TCR-induced T cell proliferation (Scharffetter-Kochanek et al, 1998), and in T cell transendothelial migration (Andrew et al, 1998 and Berlin-Rufenach et al, 1999). Interestingly, LFA-1 mutant mice having deletion of the conserved GFFKR motif in the αL cytoplasmic domain, which makes LFA-1 constitutively active, also have impaired immune responses (Semmrich et al, 2005). The phenotype was quite similar to that of LFA-1-deficient mice, including enlarged spleens and impaired lymphocyte responses, but in addition, T cell activation, cytotoxic T cell activity and T cell-dependent humoral immune responses were impaired. Thus, also deactivation of LFA-1 and disassembly of LFA-1-mediated cell contacts seem to be important for the generation of normal immune responses.

Therapeutic targeting of LFA-1

The pathology of many autoimmune diseases such as asthma, rheumatoid arthritis, multiple sclerosis (MS), thyroiditis, Crohn's disease, and insulin-dependent diabetes mellitus (IDDM) involve the infiltration of autoreactive lymphocytes to a certain organ or site in the body (Kanwar et al, 2000, Steinman, 1996, Vanderbark et al, 1996, Luster et al, 2005 and Yusuf-Makagiansar et al, 2002). The role of LFA-1 in these diseases has been extensively probed by using blocking antibodies against LFA-1 and its major ligand ICAM-1. The first LFA-1-targeted therapeutics to be tested clinically were anti-LFA-1 antibodies (odulimomab, monoclonal mouse anti-human LFA-1), which showed efficacy in clinical trials of bone marrow transplant (Fischer et al, 1986 and Stoppa et al, 1991). Using the humanized anti-LFA-1 antibody efalizumab (anti-CD11a), clinical proof for a role of LFA-1 in psoriasis has now been provided (Gottlieb et al, 2000, 2002, Lebwohl et al, 2003, Menter et al, 2005). Psoriasis is an inflammatory disease of the skin that is characterized by lymphocyte infiltration, keratinocyte proliferation, and the production of numerous pro-inflammatory cytokines and chemokines. Clinically the disease is characterized by areas of redness and skin thickening often accompanied by white scaling. The central role of T cells in the pathogenesis of this disease is well documented (reviewed by Gottlieb, 2005). In patients with moderate to severe psoriasis, treatment with efalizumab provided clear therapeutic benefit. However, in addition to binding to the LFA-1 α I domain and blocking binding to ligand, treatment with this antibody leads to dose dependent down-regulation of LFA-1 expression (Gottlieb et al, 2000 and 2002). The observed lymphocytosis in treated patients is consistent with the loss of LFA-1 function,

either through receptor occupancy and/or down-regulated expression. Such loss of LFA-1 function could prevent entry of lymphocytes into secondary lymphoid organs and/or inflammatory skin sites. Decreased expression of other potentially disease relevant molecules including, VLA-4, TCR, CD4, CD8, CD28, CD2, Mac-1 and the β7 integrin chain has also been reported (Vugmeyster et al, 2004, Gottlieb, 2005). How these changes contribute to the efficacy observed in treated patients remains to be determined. In addition to antibody-based approaches, a number of pharmaceutical companies are also actively developing small molecule antagonists of LFA-1. Further clinical testing should reveal how antibody therapeutics such as efalizumab will compare to small molecule antagonists for the treatment of psoriasis and other inflammatory diseases.

2.3 Structure and Binding

A complete understanding of integrin regulation requires knowledge of how conformational information is transmitted through the many domains that link the ligand-binding domains to the transmembrane and cytoplasmic domains. From electron microscopy (EM) studies, investigators have known for years that the overall structure of the integrins included an extracellular, globular, N-terminal ligand-binding head domain, representing a critical α and β subunit interface, standing on two long and extended C-terminal legs or stalks, which connect to the transmembrane and cytoplasmic domains of each subunit (Nermut et al, 1988). However, X-ray crystal structures of the extracellular domain of the integrin αVβ3 provided the surprising finding that the legs were severely bent, generating a V-shaped structure in which the head domain was closely juxtaposed to the membrane-proximal portions of the legs (Xiong et al, 2001, 2002). Since the determination of these initial structures, an increasing number of studies have established that the bent conformation represents the physiological low-affinity state, whereas inside-out signaling and ligand binding are associated to large-scale global conformational rearrangements in which the integrin extends (Takagi et al, 2002, Kim et al, 2003, Takagi et al, 2003, Xiao et al, 2004).

Ligand-binding head and α and β legs

Half of integrin α subunits contain a domain of about 200 amino acids known as an inserted (I) domain or a von Willebrand factor A domain. In integrins in which it is present, as in LFA-1, the α I domain is the major ligand-binding site (**Fig 3A**). Several structures of the α I domain are now available, including αL I domain with mutationally introduced disulfide bonds bound to ICAM-1, ICAM-3 and ICAM-5 (Shimaoka et al, 2003, Song et al, 2005, Zhang et al, 2008). A divalent cation-binding site, which physiologically binds Mg^{2+}, defines the top face of the domain. Divalent cations are universally required for ligand binding by integrins, and in α I domains the metal-coordinating residues and the residues surrounding the metal-binding site are important for ligand binding. Therefore, this site has been designated the metal-dependent adhesion site (MIDAS). Residues involved in ligand binding to the α I domain cluster around the MIDAS face, with an acidic residue from the ligand completing the coordination sphere of the metal ion (Emsley et al, 2000). The N-terminal region of the integrin α subunit contains seven segments of about 60 amino acids, each with weak similarity to one another. These were initially predicted (Springer, 1997) and later confirmed by crystal structures (Xiong et al, 2001, Xiao et al, 2004) to fold into a seven-bladed β-propeller domain. The upper surface of the β-propeller has been shown to contain a ligand-binding site (Emsley et al, 2000). The α I domain is

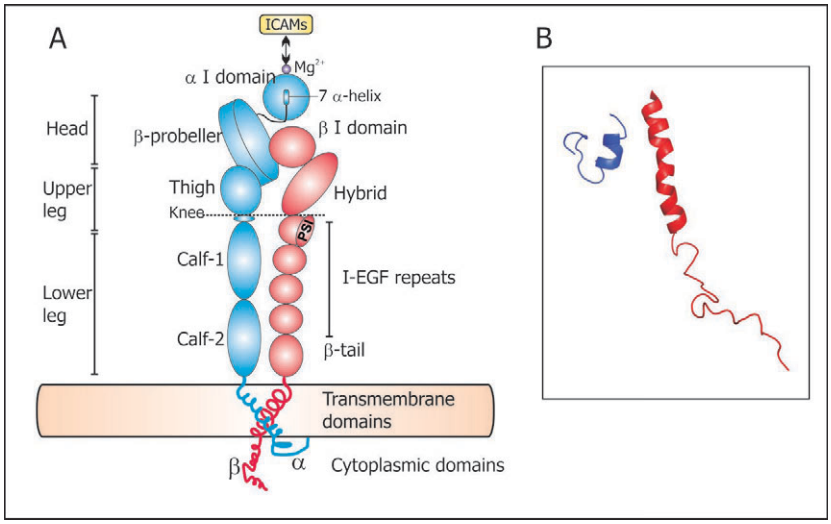

Fig 3. LFA-1 structure
A. Schematic structure of LFA-1. The α I domain binds to ICAMs in a Mg^{2+}-dependent manner at the MIDAS site. The molecule is presented in the intermediate affinity conformation. **B. Structural illustration of αIIbβ3 tail complex in solution**. Backbone ribbon diagram, where the αIIb is in blue and the β3 in red. PDB ID code 1M8O. Figure was prepared using the graphics program PyMOL.

inserted between β-sheets 2 and 3 of the β-propeller.

An inserted domain in the β subunit, the β I domain or I-like domain (Lee et al, 1995), is homologous to the α I domain, except that it contains two additional segments; one of these helps to form the interface with the β-propeller and the other is known as the specificity-determining loop (SDL) because of its role in ligand binding. One side of the β I domain binds to the upper hub of the β-propeller domain forming an extensive interface between these two domains. Mutations in the β2 I domain, that disrupt this interface, cause leukocyte adhesion deficiency (LAD) (Bilsland et al, 1994, Huang et al, 2000). Like the α I domain, the β I domain contains a MIDAS for binding negatively charged residues, which physiologically bind Mg^{2+} (Xiao et al, 2004). Additionally, there are two adjacent metal-ion-binding sites, which physiologically bind Ca^{2+}, that share some coordinating residues in common with MIDAS, and are known as the LIMBS (ligand-induced metal ion-binding site) and ADMIDAS (adjacent to metal ion-dependent adhesion site) (Xiong et al, 2001 and Xiong et al, 2002).

In the α subunit, the region C-terminal to the β-propeller comprises the leg of the α subunit and contains three β-sandwich domains. The upper leg contains the thigh domain and lower leg consists of the calf-1 and calf-2 domains. A small Ca^{2+}-binding loop located between the thigh and calf-1 domains represents the α subunit genu (knee). The β I domain is inserted in the hybrid domain, which forms the upper portion of the upper β leg. In turn, the hybrid domain is inserted to the plexin/semaphorin/integrin (PSI) domain (Xiao et al, 2004, Xiong et al, 2004 Shi et al, 2005). The rest of the β leg is built from four integrin epidermal growth factor-like (I-EGF) domains and a β tail domain. The bent structure of the αVβ3 establishes that the bend in the leg, or knee, is located between the I-EGF domains 1 and 2. The PSI and I-EGF domain 1 are located side by side. The bends in the α leg at the genu and in the β leg between I-EGF domains 1 and 2 are located close to one another and in geometry appropriate so that extension can occur by turning of the headpiece about an axis through the α and β subunit knees (Takagi and Springer, 2002 and Nishida et al 2006).

Transmembrane and cytoplasmic domains

The transmembrane (TM) segment is approximately 25-29 amino acids long. Glycosylation studies show that the TM segments are likely tilted or coiled inside the membrane (Armulik et al, 1999, Stefansson et al, 2004). Mutational studies have defined interfaces in the integrin α and β subunit transmembrane

domains that, when substituted, result in activation (Luo et al, 2004 and 2005, Li et al, 2005, Partridge et al, 2005). Modulation of the interhelical TM interface is necessary for inside-out activation, however, there is no consensus on the precise nature of this intramembranous interface or how the interface is modulated to effect activation (Adair and Yeager, 2002, Gottschalk et al, 2002, Partrigde et al, 2005, Luo et al, 2004).

The cytoplasmic tails of integrins are smaller in size (<50 residues) than the extracellular domains and are pivotal in regulating ligand binding and signaling functions of integrins (Woodside et al, 2001). Several NMR structures of integrin α and/or β cytoplasmic tails are reported mainly on αIIbβ3 integrin (Li et al, 2001, Ulmer et al, 2001, Vinogradova et al, 2000, 2002 and 2004, Weljie et al, 2002) (**Fig 3B**). NMR studies suggest that the integrin cytoplasmic tail association is weak, with significant differences between published structures of associated cytoplasmic domains (Vinogradova et al, 2002, Weljie et al, 2002) or with undetectable association between α and β subunit cytoplasmic domains (Ulmer et al, 2001, Vinogradova et al, 2004). These studies imply that the cytoplasmic interaction is modest and/or transient and that other domains are required for stable α and β association. In aqueous environment using full length cytoplasmic tails of αIIb and β3 the conserved membrane-proximal regions adopt an α-helical conformation and interact via a combination of electrostatic and hydrophobic contacts. The αIIb helix terminates at a proline residue, followed by a turn that allows the C-terminal loop to fold back and interact with the membrane-proximal region. The final 25 residues following the β3 membrane-proximal region are disordered (Vinogradova et al, 2002). Disruption of either the hydrophobic or electrostatic interactions by for example binding of intracellular proteins such as RAPL (Katagiri et al, 2003) or the talin head domain (Calderwood et al, 1999, Garcia-Alvarez et al, 2003, Tadokoro et al, 2003) to the integrin cytoplasmic tails destabilizes the cytoplasmic complex. Talin binds to the integrin cytoplasmic tail in two different steps; first it binds to the membrane distal part of the integrin tail, which becomes ordered and in the second step talin binds to the membrane proximal NPxY-motif (Wegener et al, 2007). The area upstream of the NPxY motif adopts a β strand that augments the β sheet of talin (Garcia-Alvarez et al, 2003). Filamin binding to β tail causes the β tail to form a β strand that forms hydrophobic contacts with filamin (Kiema et al, 2006). Significant changes in the structure of the full length α and β tails are also seen in the presence of a membrane-mimetic detergent. The membrane-proximal α and β segments no longer associate and the disordered C-terminal segment of β3 tail is ordered; the membrane proximal α-helix is now followed by a flexible NPxY-containing loop and a short helix (Vinogradova et al, 2004). A model was proposed in which the interacting α and β membrane-proximal regions that form in solution represent the low-affinity state of the integrin and the noninteracting tail structures in detergent represent the high-affinity integrin, in which these membrane-proximal regions are intramembranous. In another model, however, it is proposed that the interacting α and β membrane-proximal regions are embedded in the membrane in the low-affinity state, stabilized by a cytoplasmic salt bridge at their C termini and by the membrane-associated NPxY loop (Ulmer et al, 2003). Activation of the integrin breaks this salt bridge and dissociates the membrane-proximal αβ interface. This model is more consistent with data showing that the TM segment is shorter in the high-affinity state (Armulik et al, 1999, Stefansson et al, 2004).

Many studies show that deletions or mutations on the α and β subunit cytoplasmic domains, which are expected to destabilize α/β association, activate integrins (O'Toole et al, 1991, 1994, Hughes et al, 1996, Lu and Springer, 1997). Furthermore, replacement of the αL and β2 cytoplasmic domains

with acid/base peptides that form a heterodimeric α-helical coiled-coil stabilizes αLβ2 in an inactive state, whereas replacement with peptides that do not heterodimerize causes constitutive activation of αLβ2 (Lu et al, 2001b). Fluorescence resonance energy transfer (FRET) studies on live cells with αL and β2 cytoplasmic domains show that in the resting state the integrin α and β subunit cytoplasmic domains are close to on another. However, they undergo significant spatial separation upon inside-out activation and ligand binding (Kim et al, 2003).

LFA-1 ligands and ligand binding

Intercellular adhesion molecules (ICAMs) are structurally related members of the immunoglobulin superfamily (IgSF) and are the predominant ligands for LFA-1. ICAMs play a critical role in the development of the nervous system, in immune and inflammatory responses, and in embryonic development (Springer, 1990, Tessier-Lavigne et al, 1996). Five ICAM molecules have been identified thus far and are classified together because they contain two or more of a common Ig-like repeat or domain. ICAMs are all type I transmembrane glycoproteins and they have a large extracellular portion which is composed of the Ig-like domains and a relatively short cytoplasmic tail (Gahmberg et al, 1997, Cavallaro and Christofori, 2004). Although the ICAMs have structural similarities and they all bind to integrins, they still have distinct functions, cell signaling capacities and patterns of expression (Hayflick et al, 1998).

ICAM-1 is expressed constitutively only at low levels on vascular endothelial cells and on some lymphocytes and monocytes (Rohtlein et al, 1986, Patarroyo et al, 1987). ICAM-1 binds to LFA-1 through a key glutamic acid residue in domain 1 (Gahmberg et al, 1997). ICAM-1 contains five Ig-like domains and the functionally important integrin-binding site in ICAM-1 lies in the NH_2-terminal domain with some contribution by the second domain (Staunton et al, 1990, Berendt et al, 1992, Ockenhouse et al, 1992). In the structure of αL I domain bound to ICAM-1, Glu-34 at the end of domain 1 forms a direct coordination to the Mg^{2+} in the α I domain MIDAS (Shimaoka et al, 2003). ICAM-1 monomers are L-shaped because of 90° bend between domains three and four and ICAM-1 exists in its native membrane-bound and shed form as a non-covalent homodimer, and this dimerization directly correlates with enhanced binding to LFA-1 (Kirchhausen et al 1993, Miller et al, 1995, Reilly et al, 1995).

ICAM-2 consists of only two Ig-like domains, and it is expressed mainly on leukocytes and endothelial cells and in contrast to ICAM-1, ICAM-2 expression is not easily induced by commonly used cytokines (Nortamo et al, 1991, de Fougerolles et al, 1991). ICAM-2 also binds to LFA-1 through the glutamic acid residue in domain 1. The binding affinity of ICAM-2 to LFA-1 is weaker than that of ICAM-1, but stronger than ICAM-3 (Shimaoka et al, 2001). ICAM-2 has also been shown to enhance LFA-1 affinity toward its ligands in an energy, divalent cation and temperature dependent manner (Kotovuori et al, 1999).

ICAM-3 is even more restricted in expression than ICAM-2, appearing only on mononuclear and polymorhponuclear leukocytes and is proposed to be important in the initial scanning of the APC surface by T cells, and therefore, in generating the immune response (de Fougerolles and Springer, 1992, Montoya et al, 2002). ICAM-3 is closely related to ICAM-1 consisting of five Ig-domains and binding to LFA-1 trough the first domain (Fawcett et al, 1992). Although ICAM-3 is implicated in both adhesion and signal transduction events of leukocytes, it has a low affinity for LFA-1. However, this low affinity binding to LFA-1 regulates strong LFA-1-ICAM-1-mediated adhesion by driving LFA-1

into clusters to facilitate cell-cell interactions that take place in the immune system (Bleijs et al, 2000). Additionally, ICAM-3 has also been shown to be involved in costimulation of T cells (Hernandez-Caselles et al, 1993).

ICAM-4 (or LW-blood group antigen) expression is restricted to erythrocytes. It has only two Ig-like domains and ICAM-4 binds to several integrins representing different integrin families (Spring et al, 2001, Hermand et al, 2003, 2004). Recent studies show that ICAM-4-β_2 integrin interaction plays an important role in phagocytosis of red blood cells (Ihanus et al, 2007 and Toivanen et al, 2008). Deletion and mutational studies show that the first Ig-domain in the ICAM-4 is the most important in LFA-1 binding (Hermand et al, 2000).

ICAM-5 or telencephalin is solely expressed in neurons in the mammalian brain and it is structurally more complex than the others ICAMs (Gahmberg et al, 1997 and Tian et al, 2000a). Recent studies show that ICAM-5 is important in the regulation of immunological activity in the brain and in the development of neuronal synapses and signal transmission. (Tian et al, 2008, Tian et al, 2007, Nyman-Huttunen, 2006 and Gahmberg et al, 2008). ICAM-5 also binds to LFA-1 through its first domain (Tian et al, 2000).

Additionally, LFA-1 may bind to few other proteins. Interactions have been shown with E-selectin (Kotovuori et al, 1993), type I collagen (Garnotel et al, 1995) and junctional adhesion molecule 1 (JAM-1) (Ostermann et al, 2002). JAM-1 belongs to the immunoglobulin superfamily of proteins and it is important in LFA-1-dependent transendothelial migration of T cells and neutrophils and in LFA-1 mediated arrest of T cells (Ostermann et al, 2002).

2.4 Regulation of LFA-1 function

Modes of regulating integrin-mediated adhesion

After stimulation, lymphocytes acquire the ability to adhere to endothelial cells, APCs or the ECM through regulated interactions of integrins that occur in the absence of marked changes in the cell-surface expression levels of integrins. This increased adhesiveness of integrins could be brought about by affinity regulation and/or valency regulation as well as cytoskeletal interactions. Whereas affinity is associated with the physical parameters of monomeric interactions, changes in valency involve integrin diffusion and clustering in the plasma membrane. Intracellular activation signals induce a transition between different affinity states in integrins, which increases ligand binding. This process is known as affinity regulation of integrin avidity. On the other hand, clustering of integrins on the cell surface, which mediates multivalent interactions with ligands, is known as valency regulation of integrin avidity (Kinashi, 2005). Importantly, because affinity regulation and valency regulation are distinct processes, they are not necessarily exclusive and can often occur at the same time. In addition to valency regulation another affinity-independent mechanism involving interactions with and reorganization of the cytoskeleton contributes to the control of integrin-mediated adhesion (van Kooyk and Figdor, 2000, Hogg et al, 2002, Calderwood, 2004).

Affinity regulation

The arrest of leukocytes rolling on target vascular beds involves rapid responses to proper chemoattractant signals presented on the apical endothelial surface (Alon and Dustin, 2007). These chemoattractant

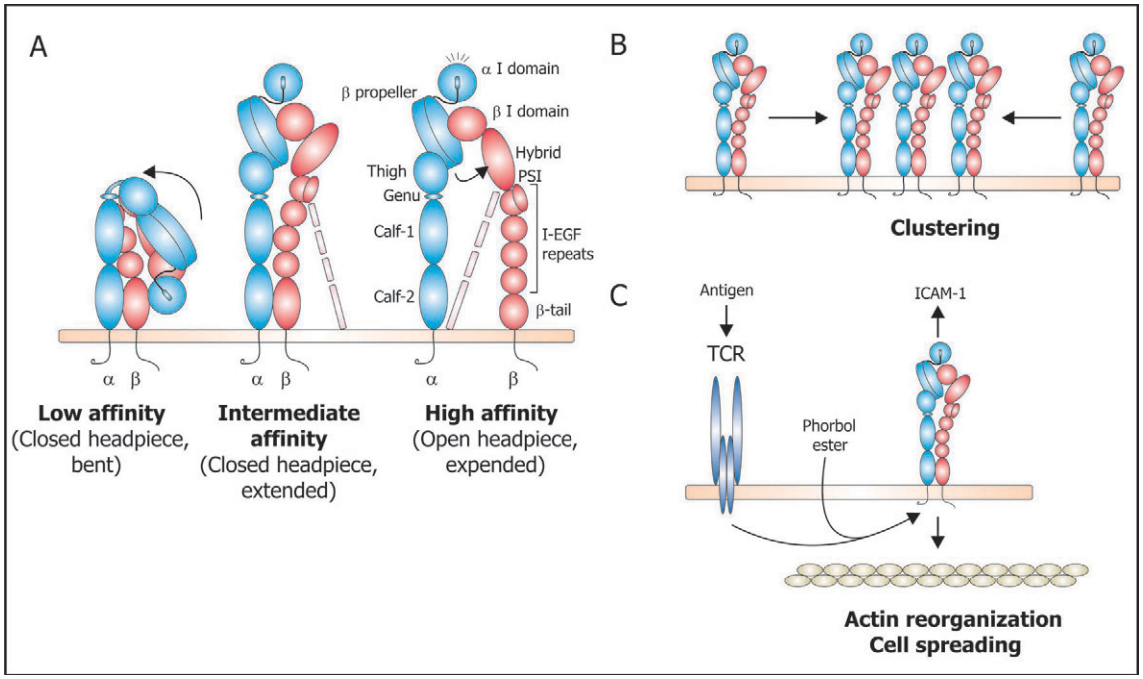

Fig 4. Modes of regulating LFA-1 function
A. Affinity regulation. The different conformations associated with distinct integrin affinities are displayed. The β subunit lower legs are flexible and are therefore shown in what may be the predominant (solid presentation) and less prominent (dashed lines) orientations. **B.Valency regulation.** Integrins cluster on the surface of a cell, which mediates multivalent interactions with ligands. **C. Cytoskeletal regulation.** Inside-out signaling through TCR ligation or phorbol ester stimulation, as well as LFA-1 ligand binding induce reorganization of the actin cytoskeleton and cell spreading.

signals convert the low-affinity integrin to the high-affinity integrin to allow firm adhesion. However, it has also been suggested that chemokines play a predominant role not in integrin-dependent firm adhesion, but rather in the adhesion during or following transendothelial migration into tissue (Carr et al, 1996). Advances in understanding integrin structure have revealed the structural basis of affinity regulation. Changes in affinity are associated with distinct conformational changes in the extracellular domains of the integrin. Structural analysis, particularly of the α I domain, has led to a general model in which conformational changes affecting integrin affinity occur after ligand binding or when triggered by inside-out signaling (reviewed by Carman and Springer, 2003 and Takagi and Springer, 2002) (**Fig 4A**). In the low-affinity conformation, the stalk region is acutely bent at the genu (knee), with the ligand binding head-piece in close proximity to the membrane-proximal stalk region. Extension of the stalk regions then shifts the molecule to higher-affinity conformations. Downward movement of the seventh α-helix (α7) of the α I domain initiates conformational change in the α I domain, altering the affinity of the magnesium-containing ligand-binding site to generate the high-affinity form of LFA-1. Engineered disulfide bonds that pull the α7 helix downward have been shown to be sufficient to induce high-affinity ligand binding (Shimaoka et al, 2000, 2001 and 2003, Lu et al, 2001a and 2001b). Thus, physiological conformational signals that exert a similar pull might function in inside-out signaling. The conformational mechanism of β I domain activation is very similar to that of α I domain. The hybrid domain exhibits two distinct orientations with respect to the β I domain in the presence and absence of ligand. In the inactive bent conformation the hybrid domain forms a restraining

interface with the leg domains, but after ligand binding the domain swings outward and pulls down the α7 helix of the β I domain, thus switching the β I domain to high affinity. The β I domain MIDAS also shifts to an open conformation upon ligand binding (Takagi et al, 2002, 2003, Luo et al, 2003, Mould et al, 2003). It is proposed that global changes in the extracellular domains generate a conformation of intermediate affinity with a closed head piece (Takagi et al, 2002, 2003) and that separation of the stalks leads to the high-affinity conformation. These conformational changes might be needed to allow cell-cell and cell-ECM adhesion, because the bent conformation of LFA-1 is predicted to extend less than 5 nm above the cell surface and therefore is likely to be topologically inaccessible to cell-surface-bound ligands (Carman and Springer, 2003, Dustin et al, 2004).

Another model for inside-out activation of integrins has also been presented. This alternate minimalist "deadbolt" model suggest, that the βTD domain and the β I domain interact and lock the integrin in the low-affinity state (Xiong et al, 2003). Modifying the TM tilt angle by inside-out signals then disengages the deadbolt, switching β I domain into high-affinity state. This model proposes that the bent conformation of the integrin can stably bind ligand. In support of this model, it has been shown that the bent conformation of the αVβ3-integrin binds to a physiological ligand in solution (Adair et al, 2005), and that the conformational change in the I domain of αL can occur independently of the conversion from a bent to an extended form (Larson et al, 2005). These data suggests that the activation of integrins is a very diverse and complex process.

Separation of the integrin α and β subunit transmembrane and cytoplasmic domains has emerged as the critical trigger for initiation of inside-out conformational signaling. Electron-microscopy studies examining mutations in the cytoplasmic regions of the integrins have shown that, in the bent, inactive conformation, there is a close association between the transmembrane and cytoplasmic regions of the α- and β-chain. Forced separation of the chains can trigger extension and conformational changes in the ligand-binding domain, generating the extended, high-affinity conformation (Lu et al, 2001, Vinogradova et al, 2002). The membrane-proximal region GFFKR motif of the cytoplasmic region of the α-chain, which constitutes the hinge domain of the α-chain, is conserved throughout all integrin-family members. This motif functions as negative-regulatory sequence by suppressing integrin activation, as indicated by the finding that deletion of this motif converts inactive LFA-1 into constitutively active LFA-1 (Hughes et al, 1996). The arginine residue in this GFFKR motif and an aspartic-acid residue at corresponding position in the β-chain are suggested to form a salt bridge, which places the cytoplasmic regions of the α- and β-chain in juxtaposition. This conformation results in low integrin activity. FRET studies in living cells have shown that separation of the cytoplasmic regions of the αL- and β2-chain of LFA-1 occurs following either inside-out signaling or ligand binding, indicating that inside-out signaling can trigger conformational changes in the extracellular regions of LFA-1 (Kim et al, 2003).

Valency regulation

Initial findings for valency regulation of integrin avidity came from the description of an anti-LFA-1 antibody that detects a Ca^{2+}-dependent epitope on LFA-1 and is only expressed when LFA-1 is in a clustered state (van Kooyk et al, 1991). Since then new evidence on the clustering of integrins has come to light. On resting cells, LFA-1 mobility is confined by cytoskeletal interactions with the cytoplasmic tail (Jin et al, 2002) but cell activation by phorbol esters or chemokines increases LFA-1 diffusion in the membrane (Kucik et al, 1996, Constantin et al, 2000) (**Fig 4B**). The lateral mobility of LFA-1 into

clusters is an important feature of the final avidity of the interaction with ICAM-1 (Stewart et al, 1998). For T cells, clustering appears to be the consequence of an initial high-affinity interaction of LFA-1 with multivalent ICAM-1 (Porter et al, 2002, Kim et al, 2004). However, in monocytes, LFA-1 pre-exists in nanoclusters that are found in lipid rafts, and these clusters enhance interactions with ICAM-1 (Cambi et al, 2006).

Even though clustering as mode of integrin regulation is a well established feature, the underlying mechanism of integrin clustering and its role in adhesion still remains poorly understood. Clustering as a mode of inside-out activation implies proactive and directed mechanisms for lateral redistribution (van Kooyk and Figdor, 2000). Vesicular trafficking (Lawson and Maxfield, 1995, Tohoyama et al, 2003) and Rap1- and RAPL-driven polarization of integrins to the lamellapodium (Katagiri et al, 2003, Shimonaka et al, 2003) represents important active modes of integrin reorganization that take place during cell migration. One proposed mechanism of clustering is based on the observation that peptides containing integrin α and β subunit transmembrane domains form homodimers or homotrimers in detergent. Thus it is proposed that homomeric association between the transmembrane domains can induce integrin clustering (Li et al, 2003). Other studies have implied a role for cholesterol-rich lipid rafts in driving integrin clustering; however, these reports are quite controversial (Leitinger and Hogg, 2002, Shamri et al, 2002, Krauss and Altevogt, 1999). The calcium-dependent neutral protease calpain has also been linked to LFA-1 avidity regulation. The expression level of calpain is increased after T cell activation and calpain inhibitors reduce the LFA-1-mediated adhesion of lymphocytes to ICAM-1 that occur after TCR ligation (Stewart et al, 1998). Calpain has been also been shown to cleave the talin-head domain from inactive talin and this active talin-head fragment regulates adhesion dynamics (Calderwood et al, 1999, Yan et al, 2001, Franco et al, 2004). Thus, the potential target of calpain in LFA-1 adhesion regulation is talin.

Cytoskeletal regulation

The relative contribution of the affinity and valency regulation to stable adhesions and the formation of T cell-APC conjunction is a subject of continued debate (van Kooyk and Figdor, 2000, Carman and Springer, 2003). However, the importance of the actin cytoskeleton for LFA-1 function is clear. The cytoskeleton is intimately involved in the dynamic regulation of the adhesive state of LFA-1 (**Fig 4C**) (van Kooyk and Figdor, 2000). The actin cytoskeleton is not only important for driving membrane remodelling, but also acts as a platform to bring together surface receptors and recruit required signaling molecules. The importance of the cytoskeleton as a dynamic regulator of leukocyte adhesion has been shown by using agents like cytochalasin D, which inhibit actin disassembly. Cytochalasin D prevents integrin clustering and subsequent adhesion. However, low levels of cytochalasin D share with phorbol esters the capacity to promote integrin-mediated adhesion (Stewart et al, 1996, Sampath et al, 1998, Lub et al, 1997 and Kucik et al, 1996). So, it is evident that the cytoskeleton is involved in the regulation of the adhesive state of LFA-1.

Disruption of the actin cytoskeleton in T cells abolishes TCR-induced binding to ICAM-coated surfaces, most likely by impairing the reinforcement of integrin-mediated contacts (Lub et al, 1997). Integrins link to the actin cytoskeleton through several proteins and have been shown to fundamentally influence actin cytoskeleton reorganization and the organization of the IS. The β subunit cytoplasmic domains are generally considered important in the regulation of integrin functions (Williams et al, 1994),

whereas in most cases the α subunit cytoplasmic domains are considered to have a more regulatory role (Calderwood, 2004). The β2 cytoplasmic domain has been shown to be essential for activation of the LFA-1 integrin (Hibbs et al, 1991a). Truncation of the β2 cytoplasmic domain after 6 amino acids from the TM domain eliminates LFA-1 binding to ICAM-1 and sensitivity to phorbol ester (Hibbs et al, 1991a). Point mutations have revealed a number of different functionally important areas within the β2 cytoplasmic domain, the cluster of three threonines (TTT 758-760) being one of them (Hibbs et al, 1991b). This threonine triplet is required for ICAM-1 binding as well as focal adhesion formation, actin cytoskeleton reorganization and cell spreading. These are so called postreceptor events which do not affect integrin affinity (Peter and O'Toole, 1995), or integrin-mediated transendothelial migration induced by chemokines (Weber et al, 1997). Phorbol ester and TCR stimulation of T cells lead to adhesion which is dependent on cell spreading and intracellular events including PKC activation, changes in intracellular Ca^{2+} concentration and actin polymerization (Stewart et al, 1996), thus implicating the importance of cell spreading and reorganization of the cytoskeleton in T cell adhesion.

Phosphorylation of LFA-1

Bidirectional signaling of integrins is likely to be regulated by the binding of different factors to their cytoplasmic domains. Because there are numerous proteins that either directly of indirectly interact with the integrin cytoplasmic tail, there must exist spatio-temporal regulation of these interactions by different mechanisms. Phosphorylation of the integrin tails is one possible regulatory mechanism and the α and β chains of LFA-1 contain several potential phosphorylation sites, and the α and β chains have been reported to be phosphorylated in cells (Fagerholm et al, 2004). Traditionally, β chain phosphorylations on serine, threonine and tyrosine residues have been much more studied than α chain phosphorylation, mainly due to the fact that the β chain phosphorylations are generally induced by stimuli that induce activation of integrins.

Phosphorylation of the β2 integrin chain occurs only after cell stimulation and several serine and threonine residues are phosphorylated (**Fig 5**). Serine phosphorylation of the β2 integrin cytoplasmic tail after leukocyte stimulation was first reported more that two decades ago (Hara and Fu, 1989, Chatila et al, 1989, Valmu et al, 1991, Chatila et al, 1988, Buyon et al, 1990, Valmu and Gahmberg, 1995) and the main phorbol-ester-induced serine phosphorylation site was found to be Ser756 (Hibbs et al, 1991a, Valmu et al, 1999b, Fagerholm et al, 2002, Hilden et al, 2003). Ser756 phosphorylation does not affect cell adhesion to ICAM-1 (Hibbs et al, 1991b), and ligation of the CD3 component of the T cell receptor does not cause phosphorylation of this site (Fagerholm et al, 2002, Hilden et al, 2003). Thus it remains to be solved whether Ser756 phosphorylation has a function in the regulation of the β2 integrin. However, this serine is well conserved in other β integrin chains and mutation in β1 affects cell adhesion and migration (Mulrooney et al, 2001).

Ser745 is not conserved in other integrins and it is a novel phosphorylation site in β2 (Fagerholm et al, 2002). It is likely to be involved in β2 integrin specific events. It has been reported that stimulation of cells by crosslinking of LFA-1 with antibodies, or by adding ICAM-2, induced phosphorylation of Ser745 (Perez et al, 2003). Phosphorylation of Ser745 leads to disengagement of the transcriptional coactivator JAB-1 (Jun activating binding protein-1) from LFA-1, which enables JAB-1 to participate in downstream signaling events. The only tyrosine residue in the β2 chain is in the membrane-proximal region of the intracellular tail. Tyrosine phosphorylation of the β2 has been speculated to be involved

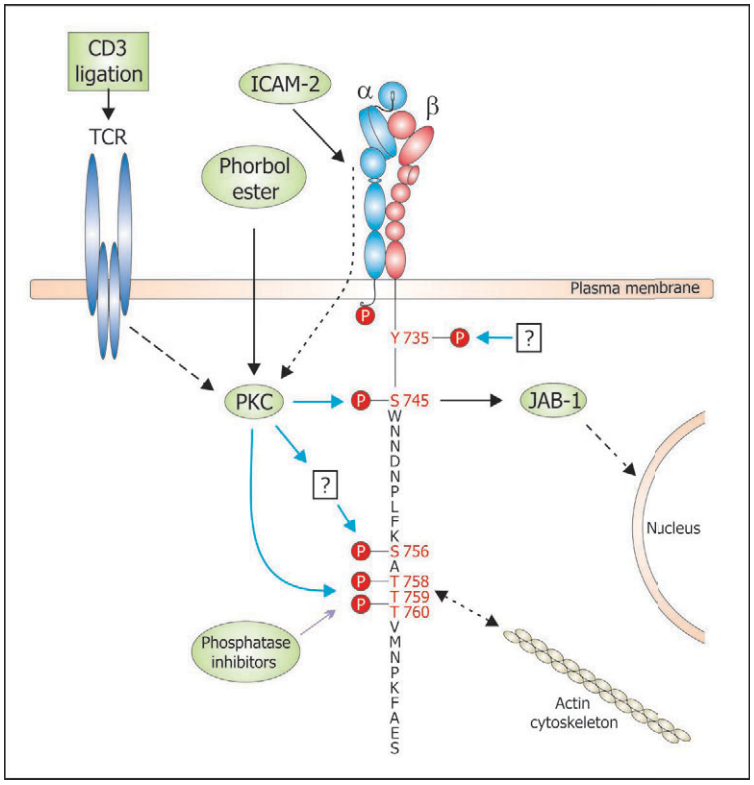

Fig 5. LFA-1 cytoplasmic tail phosphorylation.
TCR ligation induces phosphorylation of the β2 chain only at Thr758, whereas phorbol ester stimulation induces phosphorylation of Ser745, Ser756 and Thr758-760. PKC isoforms phosphorylate Ser745 as well as Thr758-760, but the kinase that phosphorylates Ser756 is unknown. Ser745 is also phosphorylated after ICAM-2 binding to LFA-1. Thr758-760 are important for integrin-mediated adhesion and cytoskeletal reorganization, but this phosphorylation is labile, and can only be detected in the presence of phosphatase inhibitors.

in outside-in signaling (Umehara et al, 1993 and Garnotel et al, 1995). Interestingly, mutation of the β2 tyrosine residue inhibits integrin recycling to the cell membrane in transfected cells, but the implication of phosphorylation in this process has not been studied (Fabbri et al, 1999).

The threonine-triplet Thr758-Thr760 in the β2 integrin cytoplasmic domain is important for integrin function. The TTT-motif is required for the interactions with the actin cytoskeleton and modulation of cell spreading and adhesion (Mastrangelo et al, 1999, Bodeau et al, 2001, Wennerberg et al, 1998, Hibbs et al, 1991a, Peter and O'Toole, 1995). After phorbol ester stimulation, all three threonine residues of the threonine triplet became phosphorylated but only two at a time. CD3 stimulation leads to a strong threonine phosphorylation of the β2 integrin, but differs from phorbol ester activation in that phosphorylation occurs only on Thr758 (Hilden et al, 2003). Threonine-phosphorylated β2 integrins distribute preferentially to the actin cytoskeleton *in vivo*, which further supports the model of the TTT-motif regulating adhesion via cytoskeletal interactions (Valmu et al, 1999). 14-3-3 proteins, which interact with threonine-phosphorylated β2 integrin peptides, might mediate the β2 integrin-cytoskeleton association (Fagerholm et al, 2002). Constitutively active protein kinase C (PKC) isoforms can induce LFA-1 binding to ICAM-1 in a model cell system (Katagiri et al, 2000). The kinases responsible for phosphorylation of the β2 integrin at Ser745 and Thr758-Thr760 were also identified as PKC isoforms (Fagerholm et al, 2002, Perez et al, 2003). The role of phosphatases in integrin regulation has not been much studied. Okadaic acid, used to inhibit protein phosphatase 1 and protein phosphatase 2A, inhibits T cell adhesion (Valmu and Gahmberg, 1995). This implies that phosphatases also have important roles in the regulation of integrin function. Although Ser745, Ser756 and Thr758-Thr760 are all phosphorylated at low stoichiometry (Valmu et al, 1999), phosphorylation of these sites regulates important protein interactions of the LFA-1.

LFA-1 α chain (αL) also becomes phosphorylated, mostly on serines. αL is constitutively

phosphorylated on serine residues in monocytes and peripheral blood mononuclear cells (Hara and Fu, 1989, Chatila et al, 1989 and Valmu et al, 1999). However, the phosphorylation sites had not been mapped, and their possible functions remained unknown.

3. LFA-1 CYTOPLASMIC DOMAIN INTERACTIONS

3.1 Important sites in the integrin cytoplasmic tails

Although integrin cytoplasmic domains are short and devoid of any enzymatic activity, these intracellular tails are vital for proper integrin function. α Chains are well conserved among different species, but they share only little homology with each other, whereas the β cytoplasmic tails show a high degree of sequence homology (Hynes, 1992). Both tails contain important motifs for regulation of integrin functions (**Fig 6**).

The β2 cytoplasmic tail

Extensive mutational analysis has demonstrated that integrin β cytoplasmic tails play a central role in the regulation integrin functions. β2, β3 and β7 integrins lacking the β tail results in a constitutively active integrin. Deletion of β2 cytoplasmic tail also alters the localization of LFA-1 into clusters and thereby regulates LFA-1 activation and LFA-1-mediated adhesion ICAM-1 (Lub et al, 1997, Hughes et al, 1995, Crowe et al, 1994). It has also been shown that isolated β tail chimeras can localize to pre-existing focal adhesions and activate downstream signaling molecules, regulate cell cycle progression and actin cytoskeleton assembly (Akiyama et al, 1994, Chen et al, 1994, LaFlamme et al, 1992, Lukashev et al, 1994, Belkin and Retta, 1998, David et al, 1999, Tahiliani et al, 1997). β Tails are thus necessary and sufficient for correct subcellular localization of integrins, for activation of signaling pathways, and for regulation of integrin affinity for their ligands (reviewed by Liu et al, 2000).

Most integrin β tails (including β2) contain one or two NPxY/F motifs (where x is any amino acid) that are part of a canonical recognition sequence for phosphotyrosine-binding (PTB) domains, which are protein modules in a wide variety of signaling and cytoskeletal proteins. Phosphorylation of the tyrosine in the NPxY/F motif may represent a mode of regulating integrin interactions with other proteins at the cytoplasmic face of the plasma membrane. However, in the β2 integrin cytoplasmic tail, both NPxY/F motifs contain phenylalanines. These phenylalanines have been shown to be important for β2 integrin activity. Phe to Ala mutations abrogates ligand-dependent adhesion, whilst Phe to Tyr mutation has no effect (Hibbs et al, 1991a and Fabbri et al, 1999). This NPxY/F motif has also been shown to be important for calcium-signaling and cytotoxic killing of target cells in response to clustering of integrin cytoplasmic domains (Sirim et al, 2001).

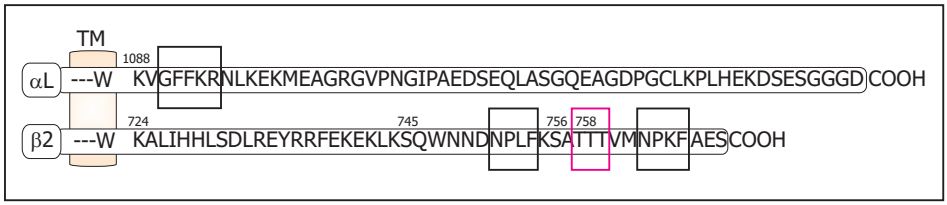

Fig 6. Amino acid sequences of αL and β2 cytoplasmic tails. GFFKR motif of αL tail and the important motifs in the β2 tail, the two NPL/KF motifs and TTT motif, are boxed. See text for more detail.

The TTT motif in the β chain is important for ligand binding (Hibbs et al, 1991a). Mutating all three threonines to alanines inhibits actin cytoskeleton reorganization and cell spreading, but does not influence integrin affinity (Peter and O'Toole, 1995) or integrin-mediated transendothelial migration induced by chemokines (Weber et al, 1997). These threonines are conserved among β1, β3 and case of β1 and β3 they have been shown to be critical for integrin function (Wennerberg et al, 1998, Stroeken et al, 2000, Bodeau et al, 2001). On the other hand the importance of the TTT-motif for β7 integrin function remains to be solved. β2 integrin-like threonine phosphorylation of the activated β7 has been observed in T cells after CD3 triggering, but this phosphorylation is not needed for β7-mediated adhesion to its ligand VCAM-1 (vascular cell adhesion molecule-1) (Hilden et al, 2003). However, another sequence in the cytoplasmic tail of β7 integrin, YDRREY (residues 735-740), has been shown to inhibit T cell adhesion to β7 integrin ligands (Krissansen et al, 2006).

The αL cytoplasmic tail

Almost all integrin α chain cytoplasmic tails contain the conserved GFF(K/R)R-motif in the membrane proximal region. This motif seems to be important in keeping the integrin in a low-affinity, closed conformation. The positively charged arginine residue and the two phenylalanine residues in the GFF(K/R)R-motif are important for maintaining the integrin in the resting state. The arginine is proposed to interact with an aspartic-acid residue at the corresponding position in the β chain forming an inter-subunit salt-bridge (Hughes et al,1996). Disruption of this interaction results in a constitutively active high-affinity conformation (Peter and O'Toole, 1995, Hughes et al, 1996, Lu, et al, 1997, van Kooyk et al, 1999, Takagi et al, 2001). Deletion of the GFF(K/R)R-motif of the αL tail has been reported to generate a constitutively active form of LFA-1 and to impair the de-adhesion from ICAM-1 which leads to defective cell migration (Semmrich et al, 2005). In contrast, signaling through LFA-1 was not affected in GFF(K/R)R-deleted cells, which implicates the importance of the β2 chain in integrin signaling.

3.2 LFA-1 – actin interactions

Correct localization of integrins and their role in cell spreading, migration and matrix assembly require connection to the actin cytoskeleton. This connection is formed by the direct or indirect association of actin-binding proteins with integrin β-tails (Calderwood et al, 2000, Critchley, 2000). The large number of integrin cytoplasmic domain binding proteins implicates the importance of the regulation of the cellular interactions (**Table 2**).

Talin

The first cytoplasmic protein to bind to integrins directly was the F-actin-binding protein talin (Horwitz et al, 1986). Talin is a 270-kDa protein, consisting of an N-terminal ~ 47-kDa globular head and a ~190-kDa, C-terminal rod domain (Rees et al, 1990). Two talin molecules form an anti-parallel homodimer. Talin plays a key role in integrin activation, the initiation of matrix adhesion formation and the linkage of integrin receptors to the actin cytoskeleton (reviewed by Arnaout et al, 2007). The integrin-binding site has been localized to the talin head, which contains a FERM (band 4.1, ezrin, radixin, moiesin) domain. The FERM domain consists of F1, F2 and F3 subdomains, of which F3 binds with the highest affinity to the cytoplasmic tails of integrin β1, β2, β3 and β5, but only weakly to β7 (Pfaff et al, 1998,

Calderwood et al, 2002). The critical F3 region contains a phosphotyrosine-binding (PTB) motif, which is known to associate with proteins containing NPxY motifs (Calderwood et al, 2002), and talin interacts in a similar way (Garcia-Alvarez et al, 2003, Wegener et al, 2007).

There are several different ways proposed how talin is recruited to the plasma membrane to the vicinity of integrins. Tyrosine phosphorylation of the β tail has been suggested to negatively regulate talin binding and integrin activation (Datta et al, 2002, Garcia-Alvarez et al, 2003). According to one theory, the proteolysis by the Ca^{2+}-dependent protease calpain releases the talin head domain from the inhibitory effects of the rod domain and allows it to interact with integrin β tails (Yan et al, 2001). Another way of releasing the talin head domain from the rod may be the binding of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). The talin rod domain specifically masks the integrin binding region in talin, and the binding of PtdIns(4,5)P2 induces a conformational change in talin, which unmasks the F3 region of the FERM domain, allowing integrin binding (Martel et al, 2001, Goksoy et al, 2008). Another candidate for modulating talin binding to integrins is the Rap1 effector RIAM (Rap1-guanosine triphosphate-interacting adaptor molecule). RIAM binds both talin and Rap1 and this binding is required for Rap1-mediated adhesion (Han et al, 2006). Recently, RIAM has been shown to be required for agonist-induced activation of αIIbβ3 by regulating talin recruitment (Watanabe et al, 2008). RIAM has also been shown to link an adapter protein ADAP (adhesion- and degranulation-promoting adaptor protein, also known as Fyb or SLAP130) and Rap1, both implicated in integrin affinity regulation downstream of the TCR (Menasche et al, 2007). ADAP may assist RIAM in localizing Rap1 and/or talin to the plasma membrane for interaction with β integrins (Lafuente et al, 2004, Lafuente and Boussiotis, 2006, Menasche et al, 2007). Finally, other PTB fold-containing proteins may also compete with talin for the NPxY/F motif, binding to integrin β tails but not activating these (Calderwood et al, 2003).

The structure of the PTB-like β-sandwich F3 in complex with the cytoplasmic tail of the integrin β3-tail has been determined (Garcia- Alvarez et al, 2003, Wegener et al, 2007) (**Fig 7B**). An NPxY motif of the β3 forms a reverse turn, with tyrosine 747 (in β3) inserting into a hydrophobic pocket in F3, an interaction stabilized by a tryptophan (W739 in β3). F3 also makes a second hydrophobic interaction upstream of the α-helical membrane proximal region of the cytoplasmic β3, which faces β-strands S1-2 and S6-7 of the F3 lobe. Lysine residues in the flexible S1-S2 loop of F3 are predicted to contact the cytosolic surface of the plasma membrane. Mutation of these lysines abolishes binding to the membrane proximal region of the β3 integrin and thus inhibits integrin activation. Thus, it is suggested that F3-membrane interactions make significant contributions to the energy required to stabilize the activated state of the integrin (Wegener et al, 2007). NMR-studies on the same complex have suggested that the second membrane proximal engagement by talin F3 disrupts the salt bridge between α and β cytosolic tails, providing a kinetic pathway for inside-out activation (Hughes et al, 1996, Vinogradova et al, 2002). Consistent with these findings is loss of fluorescence resonance energy transfer (FRET) between α/β cytoplasmic tails fused to CFP and YFP at their C-termini when activated by talin head domain (Kim et al, 2003). However, while breaking of the salt bridge seems to be necessary, it is not sufficient for full activation on its own (Tadokoro et al, 2003). In addition of talin and the integrin β tail interactions, an interaction between talin and the αIIb cytoplasmic tail has been described (Knezevic et al, 1996), but remains structurally uncharacterized. A recent model proposes that the exposed talin F3 domain binds to a membrane-distal portion of β integrin tails followed by binding to the membrane-proximal β integrin region, thereby leading to the active integrin conformation (**Fig 7A**) (Wegener et al, 2007).

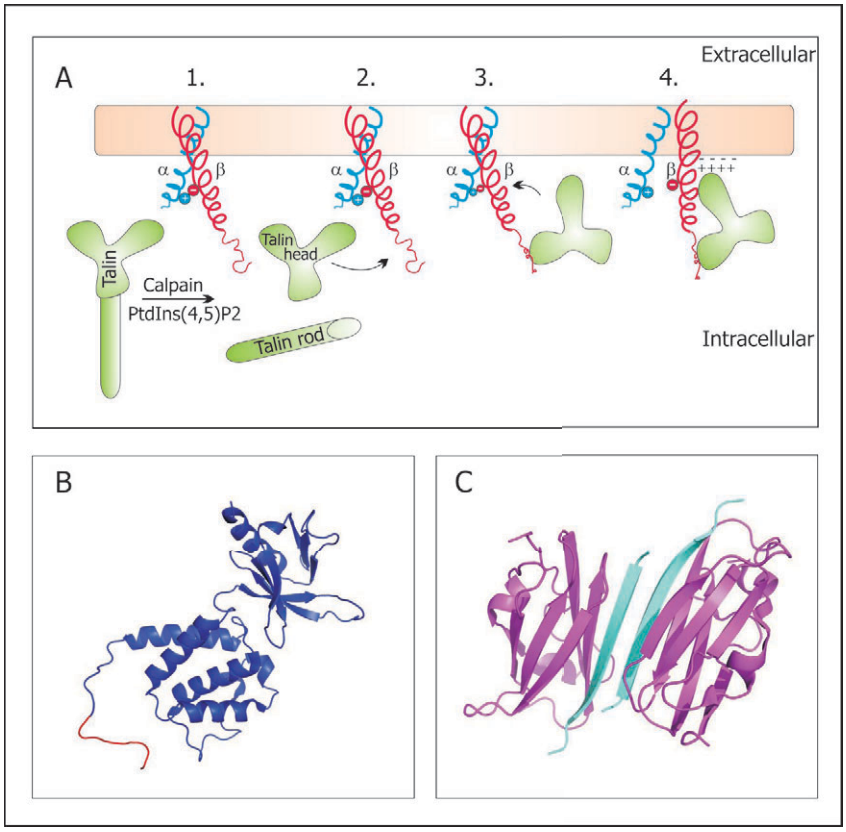

Fig 7. LFA-1 binding to talin and filamin

A. Model of talin-induced integrin activation. **1**. In a resting cell integrin tails are held in low-affinity state by electrostatic forces and the talin head domain is bound to the rod domain. Engagement of the TCR produces intracellular signals, which free the talin head domain. **2**. Talin head, and especially the F3 domain engages first the integrin membrane distal (MD) portion (NPxY motif), which becomes ordered, but the interaction between α and β that hold the integrin in the low-affinity conformation remain intact. **3**. Talin engages the membrane-proximal (MP) portion (W739) of the integrin while maintaining its MD connection. **4**. Following these interactions the putative salt bridge between α and β tails is destabilized, the helical structure of the MP region is stabilized, and electrostatic interactions between talin F3 and the acidic lipid head groups are formed. **B. Talin (F2+F3)-Integrin β3 cytoplasmic tail complex**. Ribbon diagram of talin F2+F3 domains (blue) bound to integrin β3 peptide (red). PDB ID 1MIZ **C**. **IgFLNa21-Integrin β7 cytoplasmic tail complex.** Ribbon diagram of the asymmetric unit showing two filamin molecules (magenta) interacting with two integrin β7 peptides (cyan). PDB accession code 2BRQ. Figures B and C were prepared using the graphics program PyMOL.

Whether or not this occurs in one or two separate steps, binding of F3 to both membrane distal and proximal regions is necessary (Wegener et al, 2007). Talin binding to LFA-1 has been shown to induce the intermediate affinity form of the LFA-1 integrin rather than the high affinity form (Li et al, 2007), suggesting that transition of LFA-1 across different affinity states may be required to fine-tune leukocyte adhesiveness.

The importance of talin in platelet integrin activation has been demonstrated by selectively disrupting the talin1 gene in mouse platelets (Nieswandt et al, 2007, Petrich et al, 2007). Platelets from talin1 knockout mice were unable to activate integrins in response to platelet agonists. The β1 integrin-mediated platelet adhesion as well as αIIbβ3-mediated platelet aggregation was impaired, thus establishing the crucial role of talin for the activation and function of platelet integrins *in vivo*. A very recent study also demonstrates the importance of talin-integrin interaction in fibroblast cell spreading in talin1$^{(-/-)}$ cells were talin2 was also depleted (Zhang et al, 2008). Talin was shown to provide a vital mechanical linkage between ligand-bound integrins and the actin cytoskeleton required to catalyze focal adhesion-dependent pathways.

Talin has also been shown to play an important role in chemokine-induced LFA-1-dependent adhesion and migration (Shamri et al, 2005, Smith et al, 2005). In rapidly migrating T cells high-affinity, clustered LFA-1 is restricted to a mid-cell area (focal zone) of the T cell that remains stable

over time and over a range of ICAM-1 densities. Talin is essential for the stability and formation of the LFA-1 zone. Disruption of the talin-integrin link leads to loss of zone integrity and a substantial decrease in speed of migration on ICAM-1 (Smith et al, 2005). Moreover, talin has a pivotal role in TCR-induced adhesion of T cells to ICAM-1 and in T cell-APC conjunction (Simonson et al, 2006). By depleting talin1 from human T cells using siRNA, the investigators found that both LFA-1 affinity and clustering were impaired in talin1-deficient T cells, but that rescue of affinity alone is not sufficient to rescue T cell adhesion to immobilized ICAM-1 or APCs. Thus, talin was shown to activate LFA-1 and recruit it to the site of TCR ligation, thereby stabilizing the nascent contacts between T cells and APC (Simonson et al, 2006).

Kindlins

Very recently a new protein family has been identified to be essential for integrin activation as coactivators of talin. Kindlins form a novel family of adaptor proteins that are named after the gene mutated in Kindler syndrome, an autosomal recessive genodermatosis in humans (Jobard et al, 2003). The kindlin family consists of three members; kindlin-1, -2 and -3. Kindlin-1 is expressed in epithelial cells, kindlin-2 is ubiquitously expressed and kindlin-3 expression is restricted to hematopoietic cells. The structural hallmark of kindlins is a FERM domain whose F2 subdomain is split by a pleckstrin homology (PH) domain (Ussar et al, 2006). Both kindlin-2 and kindlin-3 are vital for integrin activation as coactivators of talin (Montanez et al, 2008, Ma et al, 2008 and Moser et al, 2008). Talin is essential for integrin activation in vivo (Nieswald et al, 2007 and Petrich et al, 2007), however, talin is not sufficient for triggering the activation of integrins, but kindlins are required for the process. Both NP/XxY motifs of the β3 integrin have been shown to play important roles in integrin bidirectional signaling (Xi et al, 2003). Talin binds to the membrane-proximal motif and kindlin has been shown to bind to the membrane-distal NxxY motif. Overexpression of the talin FERM domain is sufficient to activate the αIIbβ3 integrin, whereas overexpression of kindlin-2 does not change integrin activation. But when both the talin FERM domain and kindlin-2 were overexpressed together, the talin-induced integrin activation was synergistically enhanced (Montanez et al, 2008 and Ma et al, 2008). However, open questions still remain; do all cell types and integrins need kindlins for activation, can all kindlins trigger integrin activation and can different kindlins compensate for each other. Also the mechanism behind the cooperation of kindlins and talin in integrin activation remains to be solved.

Filamin

Filamins constitute a small family of large (280 kDa) F-actin binding and crosslinking proteins (reviewed by van der Flier et al, 2001). The non-muscle filamins A and B bind to β-integrin cytoplasmic tails of β1, β2 and β7 (Sharma et al, 1995, Loo et al 1998, Calderwood et al, 2001). The interaction is strong with β7 and weaker with β1A (a splice variant of β1) (Pfaff et al, 1998). The actin-binding domain is located in the N-terminal region, while the remainder of the molecule comprises 24 Ig-like repeats, separated by two hinge regions. These domains mediate protein-protein interactions with other binding partners, and the most C-terminal repeat mediates filamin homo- and heterodimerization (Himmel et al, 2003). Apart from integrins, filamins associate with other receptors and many molecules affecting the actin cytoskeleton, such as small GTPases RhoA, Rac1 and Cdc42 (Ohta et al, 1999). Filamin binding to integrins has been reported to negatively regulate cell migration (Calderwood et al, 2001).

The crystal structure of a filamin-integrin complex has been solved and it reveals a general model for integrin-filamin interaction (Kiema et al, 2006) (**Fig 7C**). The molecular basis of the interaction was solved by using the Ig-like domain 21 of human filamin A (IgFLNa21) and the cytoplasmic tail peptide from the integrin β7. The interacting part of the β7 peptide consists of 13 residues spanning form proline 776 to proline 788, which is the stretch between two NPxY sequences. The β tail forms a β strand that lies anti-parallel to strand C of IgFLNa21. In addition to the main chain hydrogen bonding required for the β strand conformation, there are many hydrophobic contacts at the interface. On the β7 peptide, three nonpolar residues tyrosine 778, isoleucine 782 and isoleucine 786 contribute most to the interface surface area. The tyrosine 778 belongs to the first conserved NPxY motif and makes hydrophobic contacts with the C-D loop of IgFLNa21. Isoleucine 782 makes a hydrophobic interaction with the D strand of IgFLNa21 whereas isoleucine 786 forms hydrophobic interactions between strands B and C. The talin and filamin binding sites in the integrin β cytoplasmic tail are known to overlap and it has been shown that filamin and talin can compete for binding. This competition can have an impact on talin-dependent processes such as integrin activation. Phospho-mimicking glutamate residues in place of the three threonine residues in the β7 tail were shown to strongly inhibit the binding of IgFLa21, thus implicating that phosphorylation may regulate the filamin-integrin interaction, and further proposing a mechanism to control filamin binding and modulate competition between talin and filamin.

α-Actinin

α-Actinins are proteins originally discovered in skeletal muscle extracts based on their ability to bind and crosslink F-actin. α-Actinin is a homodimeric ~100-kDa rodlike protein composed of N-terminal actin-binding domain, a central region of four spectrin-like repeats and a C-terminal domain containing two calcium-binding EF hands. The non-muscle α-actinin-1 binds to cytoplasmic tails of β1, β2 and β3 integrins (Pavalko and LaRoche, 1993, Otey et al, 1990, Sampath et al, 1998, Goldmann 2000). The binding sites for α-actinin have been localized to the membrane-proximal half of the β1 and β2 integrin tail, and binding to β2 is negatively regulated by sequences in the C-terminal region of the tail (Sampath et al, 1998). α-Actinin has been shown to link actin stress fibres to focal adhesion (Rajfur et al, 2002), as well as bind to the intermediate-affinity form of LFA-1 in the leading edge of a migrating T cell (Stanley et al, 2008). This interaction has functional importance for the migrating T cell as demonstrated by α-actinin knockdown cells, which fail to migrate.

3.3 Cell signaling proteins

Outside-in and inside-out signaling are regulated by integrin β tail interactions. The absence of any detectable enzymatic activity in integrin cytoplasmic tails suggests that integrin-mediated signaling requires direct binding of signaling proteins, such as non-receptor kinases, or of adaptor proteins that can recruit these molecules (**Table 2**).

Cytohesin-1, -2 and -3

Cytohesins function as guanine nucleotide exchange factors for the ARF family of small GTPases (ARF-GEFs) (reviewed by Kolanus, 2007). The cytohesin family consists of four known members: cytohesin-1, -2, -3 and -4. These proteins share a highly similar domain organization and a relatively

small size. Cytohesin-1 is a 47-kDa modular protein with a coiled-coil N-terminus domain, which mediates interactions to cellular-binding partners. The central Sec-7 domain provides the surface for the interaction with the β2 integrin chain, and the carboxy-terminal pleckstrin homology (PH) domain is important for regulating the protein's membrane association. Cytohesin-1 and cytohesin-2 were first identified by their ability to interact with the cytoplasmic sequence of the β2 integrin chain (Kolanus et al, 1996, Korthauer et al, 2000). This interaction takes place between residues of the membrane-proximal region of the integrin β2 subunit and the Sec7 domain of cytohesin-1 (Geiger et al, 2000). The interaction between cytohesin-1 and the β2 tail results in an increased avidity of integrin-substrate binding. Since the GEF activity of cytohesin-1 is not needed for the induction of the high-affinity form of LFA-1, it indicates that protein-protein interaction alone is sufficient to regulate cytohesin-dependent inside-out signaling (Geiger et al, 2000). However, the GEF activity of cytohesin is required for cell spreading, which occurs following engagement of LFA-1 with its extracellular ligand (Geiger et al, 2000). Cytohesin-3 is also able to mediate β2 integrin adhesion (Korthauer et al, 2000), and cytohesin-2 has been shown to regulate cell motility suggesting that there may be a signaling function in which all of the closely related cytohesins-1–3 are involved (Frank et al,1998, Santy et al, 2005). Interestingly, cytohesin-1 has also been implicated in inside-out signaling through CD14/Toll-like receptor-2 in monocytes, which regulates Mac-1 (αMβ2) function, to induce adhesion and phagocytosis of mycobacteria (Sendide et al, 2005). Using siRNA-mediated knockdown, it was demonstrated that cytohesin-1 is a required component for inside-out signaling to convert the low-affinity Mac-1 to active receptor, which is then able to increase bacterial uptake (Sendide et al, 2005).

FAK

Focal Adhesion Kinase (FAK) is a 119-121 kDa nonreceptor protein kinase widely expressed in various tissues and cell types including lymphocytes. It plays a central role in signaling through integrins and a variety of other receptors (reviewed by Parsons, 2003). The kinase domain is situated in the central part of the molecule and is flanked by large non-catalytic domains. The N-terminus contains a FERM homology domain which binds to peptides of the β1, β2 and β3 integrin cytoplasmic tails (Schaller et al, 1995). The FAK binding site resides in the membrane proximal 13 residues of the integrin cytoplasmic domain, further defining the conserved aspartic acid and glutamic acid residues (D732 and E735 in β2) in the membrane proximal region essential for the interaction. The C-terminus of FAK contains a focal adhesion targeting (FAT) domain, which contains binding sites for the integrin ligands paxillin (Hildebrand et al, 1995) and talin (Chen et al, 1995). The functional relevance of direct FAK binding to integrin is unclear since the integrin-binding region of FAK is not required to target the molecule to focal adhesions (Shen et al, 1999). FAK activation in response to integrin activation leads to autophosphorylation of FAK, binding of Src family kinases and further signaling events.

14-3-3 proteins

14-3-3 proteins are a family of abundant, widely expressed 28-33 kDa acidic polypeptides that spontaneously self-assemble as dimers (reviewed by Wilker and Yaffe, 2004). They are highly conserved from plants to mammals and seven known mammalian isoforms have been identified. 14-3-3 proteins bind to numerous different proteins thus influencing several biological processes in cells (reviewed by Tzivion and Avruch, 2001). A striking feature in 14-3-3 target binding is that they almost exclusively

bind to serine and threonine phosphorylated ligands, and most of the 14-3-3 ligands contain a specific sequence (RSXpS/TXP) which allows optimal binding. The ligand binds to a specific amphipatic binding groove on the surface of the 14-3-3.

Synthetic β2 integrin cytoplasmic peptides phosphorylated on the threonine residue (Thr758) have been shown to bind to 14-3-3 proteins (Fagerholm et al, 2002). An interaction between β1 and β4 cytoplasmic tails with 14-3-3 proteins has also been reported (Han et al, 2001 and Santoro et al, 2003). However, the β1 interaction is phospho-serine and –threonine independent and the β1 integrin binds to a distinct site outside the amphipatic groove of the 14-3-3 protein (Rodriguez and Guan, 2005).

Rack1 and JAB-1

Rack1 (Receptor for activated C-kinase 1) is a 36-kDa scaffold protein containing seven internal Trp-Asp 40 (WD40) repeats (reviewed by McCahill et al, 2002). The Rack1-binding site on integrin β2 subunit resides within a conserved, membrane-proximal region. WD repeats five to seven of Rack1 (Rack1-WD5/7) interact with integrin β1, β2, and β5 cytoplasmic domains. Whereas Rack1-WD5/7 binds integrins constitutively, the association of full-length Rack1 to integrins in vivo requires treatment with phorbol esters, which promotes cell spreading and adhesion. These findings suggest that Rack1 may link protein kinase C directly to integrins and participate in the regulation of integrin functions (Liliental, et al, 1998, Besson et al, 2002).

JAB-1 (Jun-activation-domain-binding protein) is ca 40-kDa transcriptional coactivator, which binds to the β2 integrin cytoplasmic tail (Bianci et al, 2000). JAB-1 is found both in the nucleus and in the cytoplasm of cells, and a fraction of it is colocalized with LFA-1 at the cell membrane. LFA-1 crosslinking is followed by a rapid increase in the nuclear pool of JAB-1 and activation of activator protein-1 (AP-1), implicating this molecule as a mediator of outside-in signaling (Perez et al, 2003).

3.4 αL cytoplasmic tail binding proteins

The α integrin subunits play an indispensible role in integrin function, and the signaling potential of these subunits is determined by their cytoplasmic domains, which interact with intracellular accessory molecules. Like β integrin subunits, leukocyte α integrin cytoplasmic domains can interact with a variety of proteins and can be covalently modified such as by phosphorylation (Liu et al, 2000) (**Table 2**).

RapL

RapL (regulator for cell adhesion and polarization enriched in lymphoid tissue) was identified as a Rap1 effector mediating LFA-1 activation through a yeast two hybrid screen (Katagiri et al, 2003). RapL is a member of the Rassf (Ras-suppressor factor) family and consists of a central Ras-binding domain mediating Rap1 binding and a coiled coil region (Tommasi et al, 2002), which mediates the interaction of the Rap1/RapL complex with the αL subunit. The αL cytoplasmic domain amino acids K1097 and K1099 are critical for Rap1/RapL-mediated αLβ2 activation. Whether the interaction between RapLand αL is direct, is currently not known. Overexpression of RapL in leukocytes induces cell polarization and increases the binding efficiency of LFA-1 through both affinity regulation and valency regulation (Katagiri et al, 2003). RapL also regulates clustering and redistribution of LFA-1 to the leading edge of the leukocyte during polarization (Katagiri et al, 2003). Thus, LFA-1-dependent lymphocyte adhesion is

modulated by Rap1/RapL-regulated LFA-1 integrin affinity modulation and integrin clustering (Kinashi and Katagiri, 2004, Kinashi, 2005, Katagiri et al, 2003 and 2004, Miertzschke et al, 2007, Mor et al, 2007).

Binding protein	Binding site in integrin	Integrin binding site in binding protein	Effect on cells
Talin	β2, MD, NPxY motif	Head domain (F3), FERM domain	Integrin activation, cytoskeletal interaction
Filamin	β2, MP (724-747)	Ig-like domain 21	Negative regulation of migration
α-actinin	β2, MP (736-746)	?	Regulation of cell spreading and migration
Cytohesin-1, -2 and -3	β2, MP (724-725)	Sec-7 domain	Regulation of adhesion and migration?
FAK	β2, MP, (D732 and E735)	FERM-domain	?
14-3-3	β2, TTT motif	Amphipatic binding groove?	Cytoskeletal interaction?
Rack1	β2, MP	WD repeats 5-7	Links PKC to integrin?
JAB-1	β2, Ser745	?	Outside-in signaling after LFA-1 crosslinking

Table 2. LFA-1 cytoplasmic tail binding proteins. See text for references. (MD = membrane distal, MP = membrane proximal)

Calreticulin and CD45

Calreticulin, a luminal endoplasmic reticulum calcium-binding protein directly interacts with the membrane proximal KxGFFKR motif of α cytoplasmic domain (Rojiani et al, 1991). Calreticulin binds to the synthetic KLGGFKR peptide and coprecipitates and colocalizes with different α integrins (Leung-Hagesteijn et al, 1994, Coppolino et al, 1995). Calreticulin-deficient cells have a severe defect in integrin-mediated cell adhesion and, thus, the α-tail-calreticulin interaction might modulate integrin-mediated cell adhesion and signal transduction (Coppolino et al, 1997). However, the mechanism of this modulation remains to be solved.

Association of integrin αL cytoplasmic tail with CD45 (leukocyte-common antigen) in a yeast-two hybrid screen has been reported (Geng et al, 2005). CD45 is a prototypic type I transmembrane receptor-like protein tyrosine phosphatase (RPTP) with ubiquitous expression on all nucleated haematopoietic cells. The interacting region involves Glu1123–Asp1145 of αL cytoplasmic tail. This sequence is absent in αM and divergent from that of αX and αD, and may account for the specific association between CD45 and αL. CD45 and LFA-1 are reported to colocalize in the supramolecular activation cluster (SMAC) of the immunological synapse during antigen presentation (Monks et al, 1998), however, the biological function of the CD45-αL association remains to be investigated.

4. LFA-1 SIGNALING IN T CELLS

As adhesion molecules, integrins are unique in that their adhesiveness can be dynamically regulated through a process termed inside-out signaling. Thus, stimuli received by cell surface receptors for chemokines, cytokines, and foreign antigens initiate intracellular signals that impinge on integrin cytoplasmic domains and alter adhesiveness for extracellular ligands. In addition, ligand binding mediates signals from the extracellular domain to the cytoplasm in outside-in signaling (**Table 3**). These dynamic properties of integrins are central to their proper function in the immune system.

LFA-1 activation	
TCR ligation	Inside-out activation. Antigen or CD3 ligation activates TCR signaling. Influences integrin avidity and cytoskeletal reorganization.
Chemokine receptor ligation	Inside-out activation. Chemokines activate signaling downstream of the chemokine receptor to increase integrin affinity.
Phorbol esters	Inside-out activation. DAG analogs that activate PKCs directly and thus bypass TCR ligation. Influences integrin avidity and cytoskeletal reorganization.
Mg^{2+}, Mn^{2+}	Outside-in activation. Bind to α I domain MIDAS site to allow high affinity ligand binding.
Ligand	Outside-in activation. Activates signaling downstream of LFA-1 to influence cytoskeletal reorganization.
Activating antibodies	Outside-in activation. Bind to LFA-1 ectodomain to allow high-affinity ligand binding and activate signaling downstream of LFA-1 to to influence cytoskeletal reorganization..

Table 3. Different ways to activate LFA-1 integrin.

4.1 Inside-out signaling

LFA-1 mediated adhesion is induced by several extracellular stimuli in different cell types. In T-cells, LFA-1 becomes activated upon signaling from the T-cell receptor, and upon cytokine and chemokine sensing (Kinashi, 2005). Also, ligation of CD3, CD2 (Dustin and Springer, 1989, van Kooyk et al, 1989), CD44 (Koopman et al, 1990), CD45 (Spertini et al, 1991, Lorenz et al, 1994), CD98 (Suga et al, 2001), CD73 (Airas et al, 2000) and IL-2-receptor (Nielsen et al, 1996) have been shown to induce LFA-1-mediated adhesion in T cells. Because of the transient nature of integrin adhesion, the avidity of integrins must also be downregulated. One such negative regulator of LFA-1 avidity is RhoH (Cherry et al, 2004). Some of the intracellular signaling cascades controlling LFA-1 activation are discussed below.

T cell signaling in a nut-shell

The ligation of a TCR to APC by peptide-MHC and subsequent signaling is associated with the formation of an immunological synapse, a stable organization of membrane receptors, cytoskeletal components and intracellular signaling proteins (**Fig 8**).Crosslinking of the TCR initially activates

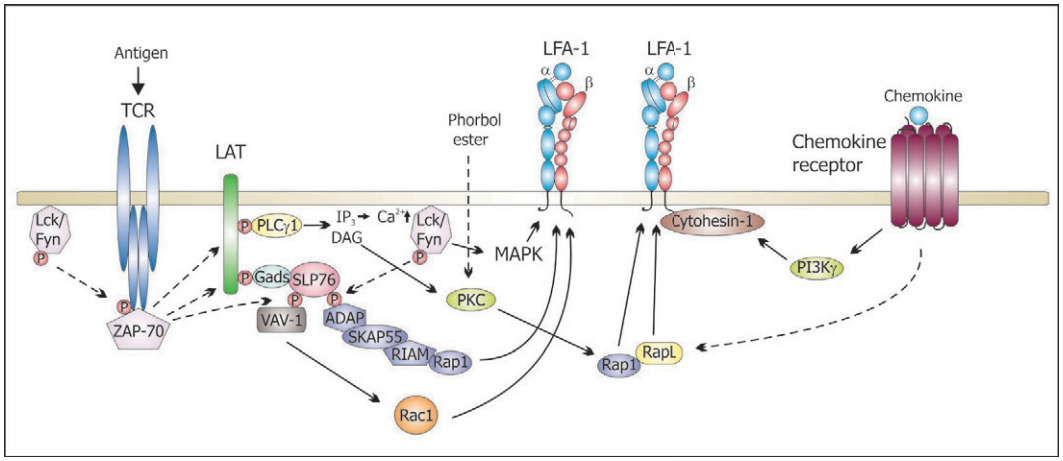

Fig 8. Intracellular signaling pathways controlling LFA-1 activation.
T cells can be activated through chemokine or TCR triggering. Ligation of the TCR leads to the activation of protein tyrosine kinases (PTK). PTKs activate PLCγ, MAPK pathway, calcium signaling and the formation of a large signaling complex to the vicinity of the plasma membrane. Chemokine triggering activates PI3-Kγ and PLCγ as well as small GTPase Rap1. All these pathways are implicated in LFA-1 regulation. See text for more details.

tyrosine kinases, for example the Src kinase Lck. Lck acts by phosphorylating immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ζ chain and also by activating a second kinase, Fyn (Friedl et al, 2005). CD3ζ phosphorylation is a hallmark of early intracellular signaling. The phosphorylated CD3ζ chain provides a binding site for the SH2 domain of the kinase ZAP70, which is then activated by Lck and Fyn (Horejsi et al, 2004). Activated ZAP70 then phosphorylates ITAMs of many substrates, most notably the membrane adapter LAT (linker for activation of T cells) (Horejsi et al, 2004, Friedl et al, 2005), thereby producing a number of attachment sites for SH2 domain-containing downstream effector molecules. These cytosolic signaling molecules include phospholipase Cγ1 (PLC γ1), SLP76 (SH2 domain-containing leukocyte phosphoprotein of 76 kDa) and phosphoinositide-3-kinase (PI3K) (Jordan et al, 2003, Horejsi et al, 2004).

SLP76 has been implicated in actin cytoskeleton remodelling (Krause et al, 2000). The binding of PI3K to LAT leads to PI3K activation. Active PI3K then phosphorylates the membrane lipid phosphatidylinositol diphosphate, leading to the formation of PIP3 (phosphatidylinositol trisphosphate). A third important molecule binding to LAT is PLCγ. It cleaves PIP2 into IP3 (inositol-tris-phosphate) and diacylglycerol (DAG). By binding to IP3 receptors in the endoplasmic reticulum, IP3 induces release of Ca^{2+} from intracellular stores into the cytoplasm (Lewis, 2001). DAG leads to the activation of protein kinase C isozymes, especially PKCθ (Friedl et al 2005). Several signaling pathways, the main ones being PI3-kinase, PKC and Rap-1, have been implicated in integrin activation (**Fig 8**). The pathways may converge in the vicinity of the integrin to regulate common downstream elements.

Pathway downstream of Src family kinases

As a result of TCR triggering and early tyrosine phosphorylation events, SLP-76 recruitment to phosphorylated LAT ultimately leads to formation of a larger signaling complex (Jackman et al, 1995, Zhang et al, 1998, Koretzky and Myung, 2001, 2006) (**Fig 8**). A haematopoietic-cell-specific cytosolic adapter protein ADAP (adhesion- and degranulation-promoting adaptor protein, also known as Fyb or SLAP130) also takes part in this complex (Kinashi, 2005). T cells from ADAP-deficient mice have

implicated the importance of ADAP in integrin inside-out signaling; ADAP-deficient lymphocytes show impaired TCR-ligation-induced β1- and β2-integrin-dependent adhesion (Griffiths et al, 2001, Peterson et al, 2001). ADAP deficiency also impairs TCR-ligation induced integrin clustering, but does not affect TCR clustering itself or assembly of the actin cytoskeleton (Peterson et al, 2001 and Griffiths et al, 2001). Moreover, SLP-76 has been implicated in the induction of LFA-1 membrane clustering and activity enhancement (Jo et al, 2005). Colocalization of ADAP with LFA-1 in the IS has also been demonstrated (Wang et al, 2004). The interaction of ADAP with SLP76 seems to be crucial for its function in LFA-1 activation. Adapter protein SKAP55 (Src kinase-associated phosphoprotein of 55kDa) might also be involved in LFA-1 activation downstream of ADAP (Wang et al, 2003). Recent data links the ADAP/SKAP55 complex and Rap1 (discussed below) to the same pathway that activate integrins following TCR engagement (Menashe et al, 2007). Following T cell activation ADAP/SKAP55 complex binds RIAM (a Rap1 effector) and this new complex provides a scaffold for the recruitment of active Rap1, thereby facilitating integrin activation.

Protein kinase C

Early tyrosine phosphorylation after TCR engagement also leads to phospholipase Cγ1-mediated hydrolysis of membrane inositol phospholipids and subsequent production of IP_3 and DAG. The second messenger DAG stimulates the activation of protein kinase C (PKC). The protein kinase C family consists of the classical serine/threonine kinases that regulate a variety of cell functions including proliferation, differentiation, cytoskeletal organization, migration, and apoptosis (Griner and Kazanietz, 2007). There are nine PKC genes, which code for isozymes classified into three groups: classical PKCs (PKCα, βI, βII, and γ), novel PKCs (PKCδ, ε, η and θ) and atypical PKCs (PKC ζ and ι) (Griner and Kazanietz, 2007). PKC is well-established intracellular effector of integrin activation as treatment of T cells with phorbol esters, cell permeable analogs of DAG, is sufficient to enhance integrin-mediated adhesion (Patarroyo et al, 1985, Dustin and Springer, 1989, Kucik et al, 1996). Inhibition of PKC activity also blocks TCR signaling to integrins (Dustin and Springer, 1989, Mobley et al, 1994). Overexpression of classical and novel PKCs has been shown to induce LFA-1 mediated adhesion to ICAM-1 and atypical PKCζ has been demonstrated to control LFA-1 lateral mobility but not affinity triggering (Giagulli et al, 2004). In addition, several different PKC isozymes have been shown to phosphorylate the β chain of LFA-1 (Fagerholm et al, 2002). A very recent study has shown that PKCθ regulates Rap1 activation in T cells (Letschka et al, 2008). A direct phosphorylation of RapGEF2 by PKCθ after T cell activation leads to the regulation of Rap1 activation as well as to the regulation LFA-1 adhesiveness to ICAM-1. It was also demonstrated that PKCθ deficiency in T cells impaires clustering of LFA-1 after antigen activation. Thus, it seems that PKCθ is needed in T cell activation by positively regulating both the cytokine responses and the adhesive capacities of T lymphocytes.

PI3-kinase pathway

PI3-kinases are a family of enzymes that catalyze the transfer of the γ-phosphate group of ATP to phosphoinositides (Wymann and Pirola, 1998), thus producing lipid phosphatidylinositol trisphosphate (PIP_3) as a second messenger. This second messenger is bound by number of proteins that contain plecstrin homology (PH) domain, recruiting these to the plasma membrane (Shimizu and Hunt, 1996, Klarlund et al, 1997). A Tec family kinase, Itk, couples PI3-K to downstream activation of LFA-1 (Woods

et al, 2001). All cell surface receptor-activated PI3-K isoforms are present in leukocytes. PI3-K α and β are activated downstream of tyrosine kinase cascades and the PI3-K γ is activated by the chemokine receptors (Sims and Dustin, 2002). PI3-K has been demonstrated to play a role in chemokine-induced clustering of LFA-1 and TCR-induced adhesion (Nagel et al, 1998, Constantin et al, 2000, Katagiri et al, 2000). In contrast, a catalytically inactive PI3K does not affect anti-CD3-induced adhesion of mouse T cells to the LFA-1 ligand ICAM-1 (Okkenhaug et al, 2002). The importance of the PI3-K pathway in integrin activation is demonstrated by PI3-K $\gamma^{-/-}$ mice. These mice show similarities in neutrophil trafficking and activation of T cells to those found in LAD patients and β2-integrin deficient mouse (Sasaki et al, 2000, Li et al, 2000, Hirsch et al, 2000). One proposed mechanism for PI3-K signaling to LFA-1 is through cytohesin-1. Cytohesin-1 binds PIP_3 and upregulates the adhesiveness of LFA-1 through valency regulation (Kolanus et al, 1996).

Small GTPases in LFA-1 signaling

The Ras superfamily of GTPases consists of small proteins that cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. They are involved in a variety of signal transduction pathways that regulate cell growth, intracellular trafficking, cell migration, and apoptosis. GPTases are regulated by GEFs (guanine nucleotide exchange factor), which control the dissociation of GDP and the entry of GTP, and GAPs (GTPase activating protein), which stimulate the intrinsic GTP hydrolysis activity of the GTPases (Scheele et al, 2007).

Members of the Rho subfamily of Ras-related GTPases play crucial roles in the regulation of the cytoskeleton in response to extracellular factors (Sorokina et al, 2005). The best studied members of the Rho family are RhoA, Rac1 and Cdc42. Activation of RhoA triggers the formation of actin stress fibers and focal adhesion complexes (Ridley and Hall, 1992a), and has also been shown to regulate chemokine-induced inside-out extension of LFA-1 before ligand binding (Pasvolsky et al, 2008 and Giagulli et al, 2004). Interfering with RhoA activity prevents the detachment of the trailing edge and dramatically reduces the rate of the migrating T cell (Mills et al, 2005). Activation of Rac1 elicits actin polymerization at the plasma membrane to produce lamellipodia and membrane ruffles (Ridley et al, 1992b) whereas activation of Cdc42 triggers the formation of filopodia (Nobes et al, 1995). In T cells, Rac1 and Cdc42 regulate spreading and adhesion to fibronectin in an integrin-dependent manner (Price et al, 1998). Moreover, cytoskeletal rearrangements and clustering of integrins at site of cell adhesion is Rac1 induced. An activated mutant of Rac1, Rac1V12, does not change the level of integrin expression or the affinity, which indicates that Rac1 might be involved in outside-in signaling to the cytoskeleton (D'Souza-Schorey et al, 1999). Vav-1 is a GEF for Rho family proteins that is highly expressed in lymphocytes. In T cells, integrin signaling leads to phosphorylation of Vav-1 and consequent spreading of T cells (del Pozo et al, 2003, Tybulewicz, 2005). The association between integrins and Vav-1 in bidirectional; Vav-1 is phosphorylated as a consequence of LFA-1 outside-in signaling, and Vav-1 transduces TCR signals, leading to the activation of LFA-1 (Tybulewicz, 2005).

Rap1 is a member of the Ras superfamily of GTPases that is highly expressed in T cells. Rap1 also undergoes GTP/GDP exchange in response to various growth factors and regulates mitogen-associated protein kinase (MAPK) cascades (Mor et al, 2007). Rap1 is activated by a number of extracellular stimuli, including TCR-ligation and chemokine and phorbol ester triggering (Katagiri et al, 2000, 2002, Shimonaka et al 2003). A deficiency of an adaptor protein Cbl-b in T cells has also been shown to

increase Rap1 activity and further enhance LFA-1-mediated adhesion of T cells to ICAM-1 (Zhang et al, 2003). The active form of Rap1 (Rap1V12) has been shown to induce inside-out changes in both conformation and affinity of LFA-1 and consistent with this is the finding that dominant-negative Rap1 blocks TCR-mediated LFA-1 activation (Katagiri et al, 2002). Moreover, T cells from Rap1-deficient mice have diminished adhesive capacity to both ICAM-1 and fibronectin, and the polarization of $Rap1^{-/-}$ T cells after CD3 stimulation is impaired (Duchniewich, et al, 2006). Inhibition of Rap1 in T cells abrogates LFA-1–ICAM-1-mediated adhesion to APCs and subsequent IL-2 production of T cells (Katagiri et al, 2002). Several molecules have been reported to interact with the GTP-bound form of Rap1. These include the Rap1 effectors, RapL, RIAM (Rap1 guanosine triphosphate-interacting adapter molecule) and PKD1 (protein kinase D1) (Menasche et al, 2007). RapL, a novel effector of Rap1 is expressed in lymphoid cells where it regulates cell adhesion and polarization. RapL associates with the cytoplasmic tail of LFA-1 after stimulation of the TCR. Overexpression of RapL augments LFA-1-mediated adhesion to ICAM-1 and a dominant-negative RapL mutant inhibits adhesion (Katagiri et al, 2003). In addition, lymphocytes from $RapL^{-/-}$ mice have defective LFA-1 dependent chemokine-triggered adhesion and migration, resulting in impaired lymphocyte homing to peripheral lymph nodes. RapL deficiency also affects both T and B lymphocyte trafficking to the spleen (Katagiri et al, 2004). RapL has recently been found to interact with the kinase Mst1 (Katagiri et al, 2006). Mst1 is required for LFA-1 clustering and adhesion to ICAM-1 and the RapL-Mst1 complex was indicated to have a regulatory function in the intracellular transport of LFA-1 (Katagiri et al, 2006). RIAM has been shown to act as a regulator of integrin activation in T cells (Lafuente et al, 2004). RIAM interacts exclusively with active Rap1 and induced cell spreading and increased β2 integrin-mediated adhesion. Unlike RapL that functions by binding directly to Rap1 and integrins, RIAM functions through an interaction with actin-regulatory proteins (Lafuente et al, 2004). PKD1 is a serine-threonine kinase downstream of PKC (Han et al, 2006). A direct interaction between PKD1 and Rap1 facilitates the activation of Rap1 and targets it to the plasma membrane (Medeiros et al, 2005). PKD1 contains a PH domain, which is critical for the activation of Rap1, since PKD1 lacking the PH domain blocks TCR-mediated activation and clustering of β1 integrins. (Medeiros et al, 2005) Whether this applies to LFA-1 and β2 integrins in general, remains to be solved.

4.2 Outside-in signaling

Binding of extracellular ligands by integrins, results in signal transduction across the plasma membrane in a process referred to as outside-in signaling, which results in reorganization and polarization of the cytoskeleton and ultimately to either stable focal adhesions, or cellular migration (reviewed by Cantor et al, 2008). The binding of ligands to the extracellular portion of integrins also leads to conformational changes in the receptors by changing interactions between the α- and β-chain cytoplasmic domains (Emsley et al, 2000) and to integrin clustering. This combination of cytoskeletal reorganization, occupancy and clustering activates several intracellular signaling pathways in cells that alter gene expression, and thus influence cell survival, differentiation, migration, morphology, etc. (Giancotti and Ruoslahti, 1999). Although integrins themselves do not have any catalytic activity, signals are transmitted through direct and indirect interactions with many partners of integrins.

The role of LFA-1–cytoskeleton interactions

Several signaling pathways activated by LFA-1 require an intact cytoskeleton (Geginat et al, 1999 and Rodriguez-Fernandez et al, 1999). One of the first molecules activated after integrin ligation is the nonreceptor tyrosine kinase FAK (focal adhesion kinase), which localizes to focal adhesions and mediates cell spreading and morphological changes (Rodriguez-Fernandez et al, 1999). In T cells the TCR-induced activation of the three main MAP kinase members (Erk, Jnk, and p38) is strongly dependent on LFA-1-dependent cell spreading and actin cytoskeleton reorganization. Furthermore, ligand engaged LFA-1 effects the activation of both Erk and Jnk and synergizes with the TCR in promoting efficient MAP kinase activation (Geginat et al, 1999). The LFA-1-mediated costimulation of cell proliferation is proposed to be due to integrin-dependent reorganization of the actin cytoskeleton and cell spreading in response to ICAM-1, which in turn influences IL-2 production and subsequent S phase entry (Geginat et al, 1999). Ligation of LFA-1 also induces actin polymerization and polarization of T cells (Kelleher et al, 1995, Rodriguez-Fernandez et al, 1999), and changes in phospholipid biosynthesis that regulates the formation and strengthening of adhesion sites, the organization and dynamics of the cytoskeleton, and cell polarity during migration (Geiger et al, 2001). Moreover, the regulation of the microtubule system at the trailing edge of a migrating T cell after LFA-1 ligation has been demonstrated to be regulated by LFA-1 (Volkov et al, 2001).

JAB-1, cytohesin-1 and ADAP as mediators of LFA-1 signaling

A transcriptional coactivator JAB-1 interacts with the cytoplasmic tail of LFA-1 (Bianchi et al, 2000). JAB-1 is found both in the nucleus and in the cytoplasm, and a fraction of it is colocalized with LFA-1 at the cell membrane. LFA-1 crosslinking is followed by a rapid increase in the nuclear pool of JAB-1 and activation of AP-1 (activator protein-1), implicating JAB-1 in LFA-1 outside-in signaling. LFA-1 has also been shown to activate PKCδ in $CD4^+$ T cells and the activation of this kinase is associated with the phosphorylation of the integrin at serine 745 of the β chain (Perez et al, 2003). This phosphorylation event is required for LFA-1-dependent c-Jun activation and subsequent AP-1 activity. Consistent with these findings, T cells stimulated with anti-CD3, anti-CD28 and LFA-1 had more AP-1 activity than cells stimulated with anti-CD3 and anti-CD28 alone (Perez et al, 2003).

Although cytohesin-1 is mainly connected to LFA-1 inside-out signaling, it is also implicated in outside-in signaling. LFA-1 stimulated Erk activation in Jurkat T cells has been demonstrated to be cytohesin-1 dependent (Perez et al, 2003). When cytohesin-1 was overexpressed in Jurkat T-cells, it associated with the plasma membrane. Treatment with ICAM-1 induced detectable threonine phosphorylation on cytohesin-1, an effect that did not occur with CD3 crosslinking of Jurkat T cells, indicating that LFA-1 signals directly through cytohesin-1. Treatment of the cells with anti-cytohesin-1 peptides inhibited Erk phosphorylation, indicating that LFA-1 can signal to both the Erk and Jnk MAPK pathways through different signaling intermediates and that one of these intermediates, cytohesin- 1, functions bidirectionally in LFA-1 signaling.

ADAP has recently also been linked to LFA-1 outside-in signaling (Suzuki et al, 2007). Direct stimulation of LFA-1 with anti-LFA-1 antibodies promotes the generation of a ring-shaped "actin cloud" that is independent of TCR activation and proximal signaling. Formation of this actin cloud is impaired following LFA-1 stimulation of ADAP-deficient T cells. Phosphorylated ADAP and clustered LFA-1 were also found in complex and ADAP coimmunoprecipitated with LFA-1 (Suzuki et al, 2007).

Thus, it is suggested that the actin cloud induced by LFA-1 engagement may serve as a possible platform for LFA-1-mediated costimulatory function for T-cell activation.

LFA-1 and costimulation

T cell activation after TCR engagement initiates intracellular signals, leading ultimately to full T cell activation. However, TCR ligation alone is not sufficient enough to fully activate the T cell, thus efficient T cell activation requires engagement of at least two types of T cell surface receptors. Costimulation signals are provided by a number of different cell surface receptors, of which CD28 is thought to be most important. LFA-1 has also been proposed to produce these costimulatory signals, although it has been difficult to distinguish between direct effects of LFA-1 engagement on T cell signaling and indirect effects mediated through intercellular adhesion (Sims and Dustin, 2002). Cross-linking of LFA-1 has been shown to activate phospholipase C, PI3K and Jnk, Pyk2, cytohesin and Erk, and JAB-1 (Kanner et al 1993, Kolanus et al, 1996, Wulfing and Davis, 1998, Ni et al, 1999, Rodriguez-Fernandez et al, 1999, Bianchi et al, 2000, Perez et al, 2003). Functionally, LFA-1 has been shown to enhance IL-2 expression leading to the induction of T cell proliferation and cell cycle progression, and to modulate effector T cell differentiation, most notably inhibiting Th2 differentiation (Bossi et al, 1998, Zuckerman et al, 1998, Shier et al, 1999). The lack of LFA-1 has also been shown to lead to profound defects in T cell signaling and proliferation (Voss et al, 1991, Scharffetter-Kochanek et al, 1998, Shier et al, 1999).

The importance of LFA-1 costimulatory signals is demonstrated by mice deficient in β2 integrin. TCR-dependent proliferation of T cells is impaired in β2 deficient mice; however, stimulation with phorbol esters resulted in normal T cell proliferation, indicating the vital role of LFA-1 costimulation in TCR-dependent proliferation of T cells (Scharffetter-Kochanek et al, 1998). LFA-1 is also a major component of the peripheral supramolecular activation cluster (pSMAC) of the immunological synapse and LFA-1-mediated formation of the pSMAC region of the IS has been shown to enhance the accumulation of TCR/class II complexes within the cSMAC region and to be critical for the exclusion of CD45 from the synapse (Graf et al, 2007). Coengagement of LFA-1 also results in a significant increase in intracellular calcium responses. Thus, LFA-1 influences TCR signaling through the organization of proteins within the immunological synapse (Graf et al, 2007).

SUMMARY OF THE STUDY

5. AIMS OF THE STUDY

The role of phosphorylation in β2 integrin signaling and in the regulation of β2 integrin cytoskeletal interaction has remained unclear. The aims of the present study were:

1. To determine the role of integrin phosphorylation in the regulation of LFA-1 activation.
2. To examine the signaling effects of β2 integrin Thr758 phosphorylation.
3. To investigate the binding of filamin, talin and 14-3-3 proteins to β2 integrins by biochemical methods and X-ray crystallography.

6. EXPERIMENTAL PROCEDURES

Detailed description of the materials and methods are found in the original publications.

Material and Methods	Original publications				
14-3-3 affinity chromatography	I	II			
Antibodies	I	II	III	IV	V
cDNA constructs	I	II	III	IV	V
Cell adhesion and aggregation assays	I		III	IV	V
Cell fractionation		II			
Co-immunoprecipitation	I	II			
Flow cytometry	I		III	IV	
Immunofluorescence staining	I		III	IV	
Immunoprecipitation	I			IV	
In vivo peritonitis model				IV	
Mice				IV	
Penetratin peptide treatment			III		
Peptide affinity chromatography	I				V
Protein crystallography					V
Protein production and purification					V
Quantitative interaction analysis					V
Rac/Cdc42 pulldown assay			III		
Radioactive cell labelling and cell activation	I				
Rap1 assay				IV	
SDS-PAGE and immunoblotting	I	II	III	IV	V
siRNA				IV	V
Soluble ligand-binding assay	I				
Synthetic peptides	I		III		V
T cell and BMDM isolation and cell lines	I	II	III	IV	V
T cell stimulation assay			III		
Transfection	I		III	IV	

7. RESULTS

7.1 The different roles of LFA-1 α- and β-chain phosphorylation in the regulation of integrin activation (I, II, IV)

Phosphorylation is a common mechanism for the regulation of surface receptor function and has also been reported in integrins (Fagerholm et al, 2004); however, its role in LFA-1 regulation has remained incompletely understood. In this study, we investigated the role of both α and β chain phosphorylation in the regulation of LFA-1-mediated adhesion. To study the phosphorylation of αL, the phosphorylation sites were first mapped. The endogenous αL was phosphorylated both in resting T cells and phorbol ester or TCR ligated T cells (I, Fig 1.) and Ser1140 being the major phosphorylation site in αL (I, Fig 1.). The stoichiometry of αL phosphorylation in T cells was determined and the immunoprecipitation studies of T cells demonstrated that approximately 40 % of surface αL is phosphorylated (I, Fig 2.). To examine the role of the Ser1140 phosphorylation site in cells, stable Jurkat Jβ2.7 cell transfectants expressing wt and Ser1140Ala mutation were generated (I, Fig 3). The binding of both wt αL and S1140A-αL transfectants to the immobilized ligand ICAM-1 was increased after TCR ligation and phorbol ester treatment (I, Fig 4.). In contrast, cells expressing S1140A-αL bound less efficiently to ICAM-1 than wt αL cells when activated by MEM-83 (an activating αL antibody) or Mg/EDTA. To support the idea that αL phosphorylation is needed for LFA-1 conformational changes, soluble ICAM-Fc binding assay and mAb24 binding (Dransfield and Hogg, 1989) were performed on wt and S1140A cells (I, Fig 4.). These experiments demonstrated that the J-β2.7 cells expressing nonphosphorytable S1140A-αL mutant showed marked reduction in ICAM-1 binding and expression of the high-affinity form of integrin. Moreover, the small GTPase Rap1 has been shown to activate LFA-1 by increasing LFA-1 affinity (Katagiri et al, 2003) and it is also known to bind the αL cytoplasmic tail. Thus, we examined the effect of active Rap1 (Rap1V12) on the wt and S1140A-mutated cells. Rap1V12 was able to induce binding of wt but not S1140A-mutant cells to coated ICAM-1.

TCR ligation and phorbol ester stimulation are known to induce phosphorylation of the β chain on Thr758 (Valmu and Gahmberg, 1995, Hilden et al, 2003), which is the first threonine in the TTT motif that is important for adhesion and cytoskeletal interactions of LFA-1 and other integrins (I, Fig7; Hibbs et al, 1991, Peter and O'Toole, 1995, Fagerholm et al, 2004). A large increase in binding of membrane proteins to 14-3-3 proteins, which bind to phosphorylated ligands, was observed after TCR ligation or phorbol ester stimulation. Using 14-3-3 affinity chromatography and immunoblotting β2 integrin and filamin were identified as a 14-3-3 protein binding partners (II, Fig 1). To establish the characteristics of this binding, we examined the binding of purified 14-3-3 proteins to the phosphorylated integrin cytoplasmic peptides. Specific binding could be observed to the phosphorylated but not to the nonphosphorylated β2 integrin peptide (I, Fig 7.). The binding was shown to be specific and occur through the canonical 14-3-3 phosphopeptide binding motif, since the 14-3-3 proteins could be eluted from the peptide affinity columns with the peptide ARAApSAPA, which specifically binds to the phosphopeptide-binding groove in 14-3-3 (Moorhead et al, 1999) (I, Fig 7.). Furthermore, endogenous 14-3-3 proteins and β2 integrins could be coimmunoprecipitated from TCR-stimulated, but importantly, not unstimulated human T cells (I, Fig 7.), indicating that these phosphorylation-dependent interactions also occur in cells.

The functional consequences of the direct association between 14-3-3 proteins and β2 integrins

were then investigated by using mutational studies. The Thr758Ala mutation, which reduces the association with 14-3-3 proteins (I, Fig 7), significantly reduced the constitutive integrin-mediated adhesion of transfected COS-1 cells to ICAM-1 (I, Fig 8.). The αL activating antibody MEM83 was still able to activate the Thr758-mutated integrin, demonstrating that activating conformational changes could still occur. Cells were cotransfected with wt LFA-1 and an EGFP-R18 construct, which blocks 14-3-3 interactions with its cellular ligands by binding to the phospho-peptide binding groove in 14-3-3 (Jin et al, 2004), to study whether the effects of the Thr758 mutation seen were attributable to the blockage of 14-3-3 proteins from binding to the integrin. Cells cotransfected with R18 exhibited markedly reduced adherence to ICAM-1 (I, Fig 8.). The TTT-motif of the integrin has been intimately associated with actin reorganization events in integrins, but not affinity changes (Peter and O'Toole, 1995). Thus we examined whether β2-14-3-3 interaction would be involved in cell spreading. The Thr758 mutation was demonstrated to significantly reduce cell spreading on ICAM-1 and in addition, the spreading of cells cotransfected with R18 and wt LFA-1 on ICAM-1 was almost completely abolished (I, Fig 9).

Talin head domain binds directly to the β chain of integrin cytoplasmic domains and, presumably, induces a separation of the cytoplasmic domains of the α and β chains, which leads to a conformational change in the extracellular domain (Kim et al, 2003 and Tadokoro et al, 2003). Thus we investigated whether talin head domain was able to activate Thr758Ala mutated β2 or Ser1140 Ala mutated αL. Talin head domain was able to induce binding of wt and both mutant COS-1 cells to ICAM-1 (I, Fig 10.), indicating that the mechanism by which phosphorylation regulated LFA-1 is not mediated by talin.

The function of Cbl-b (an adaptor protein expressed in hematopoietic cells) in LFA-1–induced cell adhesion was also examined, since T cells deficient in Cbl-b had been shown to display increased Rap1 activity and enhanced LFA-1–mediated adhesion to ICAM-1 in vitro (Zhang et al, 2003). Since we were able to show that there was an increase in LFA-1–mediated cell adhesion and spreading to ICAM-1 in Cbl-b$^{-/-}$ BMDMs (bone marrow-derived mononuclear phagocytes), it was next assessed whether Cbl-b deficiency affected LFA-1–14-3-3 interaction. In coimmunoprecipitation experiments, the association of 14-3-3β and LFA-1 β2 chain in Cbl-b$^{-/-}$ BMDMs was found to be increased compared to wt BMDMs (IV, Fig 3). In order to demonstrate that the enhanced interaction of 14-3-3β with the β2 chain of LFA-1 is responsible for the increased LFA-1–mediated adhesiveness of Cbl-b$^{-/-}$ BMDMs, the R18 construct was used to disrupt the LFA-1–14-3-3 interaction in BMDMs. The enhanced adhesion of Cbl-b$^{-/-}$ BMDMs to ICAM-1 was abrogated by the R18 peptide, which further suggests that LFA-1–14-3-3 interaction mediates the activation of LFA-1 induced by Cbl-b deficiency. Finally the increased T758 phosphorylation of the LFA-1 β2 chain was found to result from Cbl-b deficiency (IV, Fig 4). Cells lacking LFA-1 were transfected with wt LFA-1 or with T758A-β2 LFA-1 and Cbl-b was knocked down by using siRNA. Both wt LFA-1 and T758A-β2LFA-1 transfected cells behaved as previously shown, however, upon Cbl-b knockdown constitutive adhesion of wt LFA-1 transfected cells to ICAM-1 was significantly up-regulated. In contrast, Cbl-b knockdown failed to increase the constitutive or phorbol ester stimulated adhesion of mutant T758A LFA-1 transfected cells to ICAM-1 (IV, Fig 5). Thus, the T758 of the β2 chain of LFA-1 is essential in mediating the activation of LFA-1 induced by Cbl-b down-regulation.

7.2 Signaling of β2 integrin Thr758 phosphorylation in T cells (III)

We then further studied the role of Thr758 phosphorylation of the integrin β2 chain in the regulation of adhesion and signaling through LFA-1. First a phospho-specific antibody against phosphorylated Thr758 (pThr758-ab) was generated. Using this antibody the distribution of Thr758-phosphorylated integrins in detergent-soluble and –insoluble fractions from activated T cells was investigated. The Thr758-phosphorylated integrins were found both in the soluble and insoluble (cytoskeletal) fractions (III, Fig 1.), indicating that the phosphorylation does not directly regulate cytoskeletal attachment of activated integrins. Since all the functional studies performed in the previous study (I) were made with COS cells, it was of interest to see whether the same results would apply in T cells. To do this, integrin cytoplasmic peptides were coupled to activated penetratin (PENA) peptides, which are able to carry coupled peptides into cells. The effect of pThr758-β2 PENA peptide on integrin adhesion to ICAM-1 was examined and it was discovered that these cells adhered to ICAM-1 in the same manner as phorbol ester stimulated cells (III, Fig 2). This indicated that pThr758-14-3-3 complex regulates LFA-1-mediated T cell adhesion to ICAM-1. Then the possibility that the phosphorylated β2 integrin-14-3-3 protein complex might affect the activity of some enzyme was examined. Since it is known that the small GTPases Rac-1 and Cdc42 are mediators of the rearrangement of the actin cytoskeleton and that they play important roles in T cell signaling we performed a Rac/Cdc42 pulldown assay. When T cells were treated with the pThr758-β2 PENA peptide, the activation of both Rac-1 and Cdc42 was elevated ~40% (III, Fig 3). No activation of the p42/p44-MAPK pathway was observed, which indicates that the pThr758-β2 peptide does not induce a general activation of signaling pathways. To further establish the role of Rac/Cdc42 as downstream effectors of the β2-14-3-3 complex, the COS cell model was used. The constitutively active form of Rac-1 or Cdc42 was cotransfected with the T758A-mutated β2 integrin into COS cells and the adhesion of cells to ICAM-1 was observed. The constitutively active forms were indeed able to compensate for the reduced adhesion of T758A-mutated LFA-1 to ICAM-1 (III, Fig 4). The actin reorganization and cell spreading was also rescued when constitutively active Rac-1 and Cdc42 were cotransfected with mutated LFA-1 (III, Fig 4).

LFA-1 is known to act as a costimulatory receptor in T cell activation and it was of interest to study whether pThr758 was involved in the signaling events occurring after costimulation. A cell stimulation system was used, where T cell receptor antibodies (UCHT1) or control antibodies were coated onto wells and T cells treated with different penetratin peptides were then added to the wells. After 4-20h of incubation, the expression level of the CD69 receptor (an early marker of T cell activation) was measured by flow cytometry analysis. When the whole cell population was observed, the increased CD69 expression was seen in cells treated with the pThr758- β2-peptide but also in cells treated with the pαL peptide, indicating a role for phosphorylated αL in T cell stimulation (III, Fig 6). A lower effect could also be seen with the nonphosphorylated β2 peptide but the penetratin peptide alone did not induce any CD69 expression. However, when the percentage of activated cells was examined, it was found that the pThr758- β2 peptide and pαL peptide both increased the amount of activated cells when used together with UCHT1, whereas the nonphosphorylated β2 peptide and penetratin peptide alone could not increase the amount of activated cells (III, Fig 6).

7.3 The effect of Thr758 phosphorylation of the LFA-1 on 14-3-3 protein, filamin and talin binding (V)

The region in the β2 integrin cytoplasmic tail that binds 14-3-3 proteins is known to interact with filamin in other integrins (Calderwood et al, 2001), and filamin has also been reported to associate with β2 integrin *in vivo* (Sharma et al,1995, Valmu et al, 1999) and with the β2 integrin peptide containing the TTT sequence *in vitro*. In this study, we investigated the binding of the β2 integrin cytoplasmic domain to filamin, talin and 14-3-3 proteins. First, the effect of phosphorylation of Thr758 on 14-3-3, filamin and talin binding to β2-cytoplasmic peptide was characterized. 14-3-3 proteins from T cell lysates were found to interact only with the pThr758 peptides and not at all with unphosphorylated or T758A mutated β2 integrin peptide (V, Fig 1). In contrast, filamin from T cell lysates interacted well with the unphosphorylated peptide, but binding was significantly weaker to the phosphorylated peptide (V, Fig 1). As any full-length talin binding to the integrin cytoplasmic peptides could not be detected, the purified talin F2/F3 domains were used. Phosphorylation of Thr758 did not influence talin binding to the integrin cytoplasmic tail peptides, but the phosphorylation indirectly influences talin binding, as association of 14-3-3 led to detachment of talin from the phosphorylated integrin peptide (V, Fig 1). This result suggests that 14-3-3- and talin-binding sites in the integrin are partially overlapping.

To measure the affinity of the studied protein-protein interactions, we performed surface plasmon resonance (Biacore) measurements. Phospho- and unphospho-β2 peptides were coupled to Biacore sensor chips, and different concentrations of 14-3-3ζ, IgFLNa21, or talin F2/F3 were added to the solution. The 14-3-3 protein interacted only with the phospho-β2 peptide with high binding affinity (Kd 260 nM) (V, Fig 2).Talin F2/F3 interacted with similar affinity with both the unphospho- and the phospho-β2 peptide with K_ds 11 and 12,5 nM, respectively (V, Fig 2). The main integrin-binding site of filamin A, immunoglobulin domain 21 (IgFLNa21), interacted only with the nonphosphorylated β2 peptide, and the interaction was considerably weaker that for the 14-3-3 and talin interactions, in the range of 0,5 mM (V, Fig 2). It was further determined that filamin binding to β2 integrin is regulated by phosphorylation rather the 14-3-3 binding (V, Fig 2).

To understand the structural basis of the β2 integrin cytoplasmic domain binding to filamin and 14-3-3, the β2 integrin peptide with IgFLNa21 was cocrystallized and the β2 phosphopeptide was soaked in crystals of 14-3-3ζ. The IgFLNa21-β2 peptide crystals diffracted to 2.2-Å resolution. The structure was solved by molecular replacement using the previously solved IGFLNa21 (PDB ID 2BRQ) as the search model V, Fig 3). The β2 peptide bound to IgFLNa21 forming a hydrogen-bonded β-strand next to the strand C of IgFLNa21 and interacting hydrophobically with the side chains of strand D (V, Fig 3). The main hydrophobic contacts were mediated by the side chain methyl groups of Thr758 and Thr760 of the β2 integrin. Thus, the structure of the IgFLNa21-β2 peptide complex explains why Thr758-phosphorylated β2 integrin is not able to interact with filamin. The 14-3-3ζ crystals diffracted to 2,5-Å resolution. The diffraction data indicated that the best crystals were merohedral twins with twinning fraction of 0,306. The soaked β2 phosphopeptide was bound to all 4 14-3-3ζ monomers of the asymmetric unit, and the β2 phosphopeptide was bound to the well-characterized binding pocket between α-helices E and F of each 14-3-3 molecule. The main electrostatic interactions were between the Thr758 phosphate group of the β2 peptide and 14-3-3ζ Arg residues 56 and 127, which form a typical basic batch in the binding site (V, Fig 4). Also a hydrogen bond was observed between the phosphate group and Tyr128 of 14-3-3ζ and another between the side chain of Ser756 and Trp228 and

Glu180 of 14-3-3ζ (V, Fig 5). The interaction features between the β2 phosphopeptide and 14-3-3ζ are similar to other published structures where the phosphate group is the key determinant for binding.

Finally, to investigate the role of filamin binding in regulating β2 integrin-mediated adhesion to ICAM-1, knockdown experiments using siRNA of filamin were performed. Filamin knockdown did not influence basal adhesion to ICAM-1; however, it increased binding of phorbol ester stimulated Jurkat T cells to ICAM-1 (V, Fig 6). These results indicate that filamin plays an inhibitory role in regulating T cell adhesion to ICAM-1.

8. DISCUSSION

8.1 Serine phosphorylation of the αL chain regulates LFA-1 activation through affinity- dependent mechanisms

Phosphorylation of the cytoplasmic domains of LFA-1 has been reported almost two decades ago; however, its role in the regulation of integrin function has remained poorly understood (reviewed by Fagerholm et al, 2004). The phosphorylation of the αL cytoplasmic tail of LFA-1 is known to be constitutive and we were able to map the phosphorylation site to Ser1140 and the stoichiometry of the phosphorylation was also determined; 40 % of the surface αL was shown to be phosphorylated in T cells. Integrins have been shown to undergo a global conformational change from bent form to an extended form upon cell activation, and for optimal interaction between LFA-1 and ligand, the α I domain must go through critical rearrangements by pulling down the C-terminal α7 helix (Shimonaka et al, 2002). By mutating the Ser1140 to a non-phosphorylatable amino acid, we showed that phosphorylated Ser1140 is involved in conformational changes in LFA-1 in response to several different affinity-increasing stimuli, such as ligand or chemokines, or an activating antibody. The S1140A mutated integrin was able to undergo the conformational change from bent to extended conformation similar to that of wild-type cells, as detected by the KIM127-antibody which detects the extended form of LFA-1 (unpublished data), indicating that the αL phosphorylation is involved in the α I domain rearrangements Interestingly, using the KIM127-antibody with αM S1126A mutated β2 integrin Mac-1, it has been demonstrated that the mutant Mac-1 integrin extracellular domain is incapable of undergoing correct global conformational changes (Fagerholm et al, 2006). One possible mechanism for the involvement of αL in α I domain rearrangement, was that the negative charge induced by phosphorylation facilitates the separation of the integrin cytoplasmic tails, which has previously been shown to lead to a conformational change in the extracellular domain (Kim et al, 2004, Adair et al, 2005). However, it should be noted that the mutation of Ser1140 to phosphorylation mimicking Asp did not lead to a different phenotype compared to the S1140A mutation.

The α4 serine phosphorylation has been shown to inhibit paxillin binding, and further regulate cell migration (Han et al, 2001, 2003). Thus, it is conceivable, that the αL phosphorylation site could also act through cytoplasmic binding proteins that selectively bind to it or on the other hand disconnect them from the phosphorylated form of the integrin. We indeed demonstrated that active Rap1 (Rap1V12) was unable to induce binding of S1140A-mutant cells to ICAM-1, suggesting that Rap1-induced activation requires αL phosphorylation. It is possible that Rap1 influences the binding of some cytoplasmic factor to the phosphorylated αL tail. Several molecules have been reported to interact with the GTP-bound form of Rap1. These include the Rap1 effectors, RapL, RIAM and PKD1 (Menasche et al, 2007). RapL has also been shown to associate with the cytoplasmic tail of LFA-1 after stimulation of the TCR (Katagiri et al, 2003), whereas RIAM functions through an interaction with actin-regulatory proteins (Lafuente et al, 2004). It is of future interest to study the effects of these Rap1 effectors in S1140A cells. The α chain phosphorylation on Ser1126 of another β2 integrin, Mac-1, has also been reported to play crucial roles in leukocytes (Fagerholm et al, 2006). Mutation of the Ser1126 to Ala abolished binding to ICAM-1 and -2 and further inhibited chemokine-induced migration in vitro as well as significantly reduced the accumulation of intravenously administered cells in the spleen and lungs of Balb/c mice. These results demonstrate that the α chain phosphorylation of β2 integrin also plays vital roles in integrin regulation

and leukocyte migration in vivo.

8.2 β2 integrin–14-3-3 protein complex regulates LFA-1 function through cytoskeletal rearrangements and mediates signals in T cells

The phosphorylation of the β2 tail threonine triplet (TTT 758-760) occurs only after cell stimulation by TCR ligation or phorbol ester treatment (Valmu and Gahmberg, 1995). This TTT motif has been demonstrated to be important for both adhesion and cytoskeletal reorganization mediated by LFA-1 (Hibbs et al, 1991, Peter and O'Toole, 1995). The ligation of the TCR and treatment with phorbol esters has also been shown to induce clustering of integrins on the cell surface rather that affinity changes (Stewart et al, 1998). Moreover, the threonine phosphorylated LFA-1 has been shown to distribute preferentially to the actin cytoskeleton (Valmu et al, 1999), indicating cytoskeleton-dependent mechanisms of integrin activation. The linkage between integrin and the cytoskeleton can occur via actin-binding proteins or through adaptor proteins

14-3-3 proteins, which bind to serine- and threonine-phosphorylated ligands, have previously been shown to bind β2 integrin peptides phosphorylated on Thr758 (Fagerholm et al, 2002). We were able to show that this interaction between β2 integrin and 14-3-3 proteins also occurs in cells and the binding is direct and Thr758 phosphorylation-dependent. The binding is only observed in T cells after cell stimulation which leads to phosphorylation of Thr758. Mutation of Thr758 or blocking the 14-3-3-β2 interaction by an R18-construct inhibited LFA-1-mediated cell adhesion to ICAM-1 as well as cell spreading. Thus, phosphorylation of Thr758, which is induced by inside-out activating stimuli for the integrin, mediates binding to 14-3-3 proteins in cells, and this interaction seems to mediate the effect of the integrin on the cell cytoskeleton.

The 14-3-3 proteins are dimers and both monomers can independently bind to phosphorylated targets either within the same protein or different proteins (Tzivion and Avruch, 2002, MacKintosh, 2004). Thus, it is possible that threonine phosphorylation of β2 recruits 14-3-3 proteins to the plasma membrane-cytoskeleton connection and that the 14-3-3s in turn recruit other proteins to regulate downstream events. We were able to demonstrate that Thr758 phosphorylated LFA-1 integrins are enriched in, but not exclusively restricted to, the cell cytoskeleton, indicating that a simple cytoskeletal linking function for the Thr758 phosphorylated β2-14-3-3 complex is not probable. However, the complex could influence adhesion in some other way, for example by linking phosphorylated integrins together to induce clustering. However, using cell permeable penetratin-coupled Thr758-phosphorylated β2-peptides on T cells, we discovered that these peptides stimulated adhesion to ICAM-1, implying that the Thr758 phosphorylated β2 integrin might have a signaling role in T cells. The small GTPases Rac-1 and Cdc42, which have previously been implicated in integrin 14-3-3 signaling (Bialkowska, et al, 2003) and LFA-1 activation (Katagiri et al, 2000), were possible candidates as downstream mediators and we were able to show that penetratin coupled pThr758 β2 peptides activated both Rac-1 and Cdc42 in human T cells and that the active form of these GTPases, but not RhoA, were able to compensate for the T758A mutation of the β2 integrin in cell adhesion assays. Furthermore, RhoA has been shown to be downstream of the β2 threonine triplet in Mac-1 (αMβ2)-mediated monocyte phagocytosis (Wiedemann et al, 2006), implicating that small GTPases of the Rho family are essential downstream effectors of the leukocyte integrins in several β2 integrin-mediated functions and that the functionally important threonines in β2 mediate the activation of these GTPases.

14-3-3 proteins bind many proteins involved in the regulation of small GTP-binding proteins in cells (Jin et al, 2004). For example Rac-1/Cdc42 guanine nucleotide exchange factor (GEF) has been shown to bind 14-3-3 proteins, and thus it is conceivable that a phospho-β2–14-3-3– GEF -complex works on the small GTPases in T cells, thereby mediating their activation. Indeed, LFA-1 activation in T cells by Mn^{2+}, clustering or with direct mAbs has been demonstrated to induce a potent and transient activation of Rac-1, which is regulated by Vav1 (Rho/Rac family GEF) controlling the activation phases of Rac-1 activity (Sanchez-Martin et al, 2004). Vav1 activation and consequent Rac-1 activity was observed to lead to changes in cell morphology and motility (Sanchez-Martin et al, 2004). In response to TCR ligation, Vav1 has been shown to undergo rapid tyrosine phosphorylation (Margoli et al, 1992, Gulbins et al, 1993). Thus, it does not seem conceivable that after TCR ligation 14-3-3 proteins would form a complex with phospho−β2 integrin and Vav1, since 14-3-3 proteins bind to phosphorylated threonine/serine motifs and not phosphorylated tyrosine motifs. However, 14-3-3 proteins may bind to some other Rho/Rac family GEF which in turn activates Vav1 and Vav1 further activates Rac-1 and Cdc42 and this ultimately leads to actin reorganization and cell spreading. This kind of activation of Rho/Rac GEFs by another GEF of the same family has previously been reported (Vigorito et al, 2003). One possibility might also be that phospho-β2-integrin− 14-3-3 complex activates the kinase that phosphorylates Vav1, since 14-3-3 has been shown to bind to several different kinases (Jin et al, 2004). However, the kinase responsible for Vav1 phosphorylation is at present not known (reviewed by Hornstein et al, 2004). It is also possible that 14-3-3 proteins do not bind two different ligands when mediating the effects to the cytoskeleton. In this case it is conceivable that the function of 14-3-3 protein is merely to outcompete talin for β2 integrin binding and thus contribute to the initiation of further downstream signaling cascades and for the formation of a mature IS.

The effect of phosphorylated integrin on T cell-mediated functions was further approached by studying the signaling function of the phosphorylated β2 integrin in T cell costimulation. LFA-1 has been clearly demonstrated to act as a costimulatory molecule in T cell activation (Geginat et al, 1999, Ni et al, 1999). Using a T cell stimulation system and the pThr758-β2 penetratin peptides, we showed that administration of this peptide to T cells induces further downstream signaling (expression of the T cell activation marker CD69) in a similar manner to coated TCR antibody together with ICAM-1. The LFA-1-ICAM-1 interaction has previously been shown to induce Rac-1 activation in T cells (Sanchez-Martin et al, 2004), and Rac-1 has been implicated in the up-regulation of CD69 expression in T cells (Villalba et al, 2000), thus indicating that the costimulatory effect of the pThr758-β2 penetratin peptide may be due to Rac-1 activation. The large amount of phosphorylated integrin peptides introduced into cells by penetratin may mimic the clustering of LFA-1 integrins induced by ICAM-1 in other stimulatory systems leading to Rac-1 activation (Sanchez-Martin et al, 2004).

8.3 Thr758 phosphorylation of the LFA-1 β2 tail regulates the binding of cytoskeletal proteins

Next we studied the mechanism by which the Thr758 phosphorylation might regulate the binding of different cytoskeletal proteins to the β2 integrin tail, and could this regulation affect the hierarchy of the binding. The region in the β2 cytoplasmic tail that binds to 14-3-3 proteins is known to interact with filamin in other integrins (Calderwood et al, 2001). Filamin has also been reported to associate with β2 integrins in vivo (Sharma et al, 1995) and bind to β2 integrin peptide containing the TTT sequence in vitro (Valmu et al, 1999). This interaction between filamin and β2 integrin has been demonstrated to negatively regulate talin binding and talin-dependent integrin activation (Kiema et al, 2006) as well as cell migration (Calderwood et al, 2001). We demonstrated that the phosphorylation of β2 integrin on Thr758 leads to impairment of filamin interaction and binding of 14-3-3, and that 14-3-3 can outcompete talin for binding to the β2 integrin in its phosphorylated state.

Phosphorylation of the β2 tail negatively regulates filamin binding as demonstrated by the structure of the IgFLNa21-β2 integrin peptide complex. The main hydrophobic interaction with the Phe2285 of the IgFLNa21 is mediated by Thr758 in β2 integrin. The methyl group of Thr758 points toward the interface, whereas the hydroxyl group is partially exposed. Based on the structure, it is apparent that the addition of a phosphate group to Thr758 would be very unfavourable for the β2-IgFLNa21 interaction. This was also demonstrated by using phosphopeptide affinity chromatography and surface plasmon resonance assays. Thus, filamin was shown to bind to β2 integrin tail only when it is not phosphorylated, whereas 14-3-3 proteins bind to β2 integrin when the integrin is phosphorylated on Thr758. The crystal structure of the pThr758β2-14-3-3 complex revealed that the phosphate group is the major determinant of the interaction. We were also able to show that knocking down filamin by siRNA leads to an increase in stimulated T cell adhesion to ICAM-1. Thus, filamin binding negatively regulates integrin activation until TCR ligation leads to Thr758 phosphorylation and to the detachment of filamin from β2 integrin tail. These results indicate that Thr758 phosphorylation is an important molecular switch that regulates filamin and 14-3-3 binding.

Another actin-binding protein, α-actinin, has also been reported to interact with LFA-1, although we could not detect binding of α-actinin to any of the β2 integrin cytoplasmic tail peptides. It has been shown to bind at the membrane proximal site of the β2 tail, whereas the C-terminal portion of the tail, including the TTT motif, (residues 748-762) inhibits this interaction (Sampath et al, 1998). However, substitution of Thr758 with Ala or phosphate-mimic Glu stimulates binding of α-actinin to β2, suggesting that phosphorylation of β2 on Thr758 does not regulate α-actinin binding. In addition, deletion of the α-actinin binding site in β2 does not influence adhesion to ICAM-1(Sampath et al, 1998).

The interaction of the integrin activator talin with β2 integrin cytoplasmic tail was also examined. The β2 integrin has been shown to bind to the head domain of talin (Horwitz et al, 1986) and induce the separation of the α and β chain cytoplasmic domains. The talin-binding site in the β3 integrins encompasses the first NPxY motif (Wegener et al, 2007) and the corresponding residues in the β2 are 747-755, which precede Thr758. As expected since the talin binding site does not overlap with Thr758, we were able to show that the T758A mutation does not influence the activation of the integrin induced by talin head domain, indicating that T758A mutation does not affect how talin activates the integrin. We also found that phosphorylation of the Thr758 does not directly influence the binding of talin to the integrin. However, it appears that 14-3-3 binding to the phosphorylated integrin may

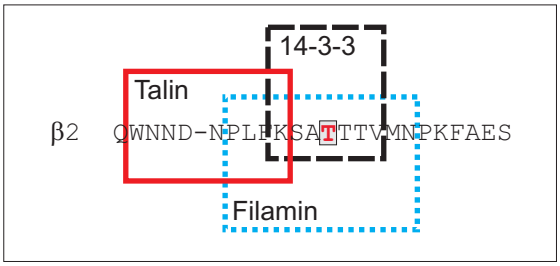

Fig 9. The binding sites of talin, filamin, and 14-3-3 protein in β2 integrin cytoplasmic tail.

lead to the detachment of talin from the integrin tail. The 14-3-3 binding to the phosphorylated Thr758 and simultaneous displacing of talin might be the needed step in integrin downstream signaling, Rac-1/Cdc42 activation and ultimately actin reorganization and cell spreading. The talin binding site in the β3 integrin (Garcia-Alvarez et al, 2003) and the 14-3-3 binding site in β2 show only one amino acid overlap (**Fig 9**), but that might be enough to prevent the simultaneous binding of these two molecules to phosphorylated integrin tails. Moreover, talin binding to the β3 integrin tail has been shown to be negatively regulated by tyrosine phosphorylation to Tyr747 of the membrane proximal NPLY motif (Oxley et al, 2008).

Phosphorylation of the Tyr757 also acts as switch to change the binding preference of the integrin tail between two different proteins, talin and Dok1 (downstream of kinase signaling protein). Talin binds with high affinity to the unphosphorylated NPLY motif and activates the integrin, whereas phosphorylation of Tyr747 enhances binding to Dok1 ~400-fold and this binding negatively regulates talin binding. Thus, tyrosine phosphorylation of the β3 cytoplasmic tail allows Dok1 to compete strongly with talin, and this ultimately results in down-regulation of integrin activation (Oxley et al, 2008). Taken together, phosphorylation of the β integrin tails is a vital mechanism in regulating the binding of intracellular proteins to integrin tails. Thus it provides an elegant mechanism to regulate the activity of the integrins and further the adhesion to integrin ligands.

8.4 Conclusions

When combining the results of this thesis one could envision a sequential model by which the proteins examined in this study influence LFA-1 integrin-mediated adhesion (**Fig 10**). In resting T cells, when β2 integrin is unphosphorylated, filamin is bound to the integrin and plays an inhibitory role in integrin activation. Talin is directly involved in β2 integrin activation by regulating both integrin affinity and clustering (Simonson et al, 2006), and talin and filamin may also compete for the binding to β2 integrin, as has been described for the β7 integrin (Kiema et al, 2006). The importance of talin in β2 integrin-mediated cell-cell binding is especially pronounced at early stages (30 seconds to 5 minutes) after T cell receptor stimulation, although whether there is a constitutive or stimulated association between talin and LFA-1 remains to be solved (Simonson et al, 2006). The binding motif NPxY for talin in β2 cytoplasmic tail contains a phenylalanine instead of a tyrosine which indicates that talin binding to β2 tail can not be regulated by tyrosine phosphorylation as is the case for β3 integrin. It is conceivable that the binding of 14-3-3 proteins to phosphorylated β2 tail may serve the same purpose in β2 integrin as tyrosine phosphorylation for β3 integrin (Oxley et al, 2008). 14-3-3 does not bind to the integrin until the β2 chain becomes phosphorylated at Thr758, which has been reported to take place at later stages of TCR stimulation (5-10 minutes) (Valmu and Gahmberg, 1995). At these later time points, the contact between T cell and APC matures and talin is no longer crucial for adhesion (Simonson et al, 2006). Thus, phosphorylation of Thr758 directly inhibits filamin binding, and indirectly inhibits talin binding to the β2 integrin as 14-3-3 proteins displace talin after Thr758 phosphorylation.

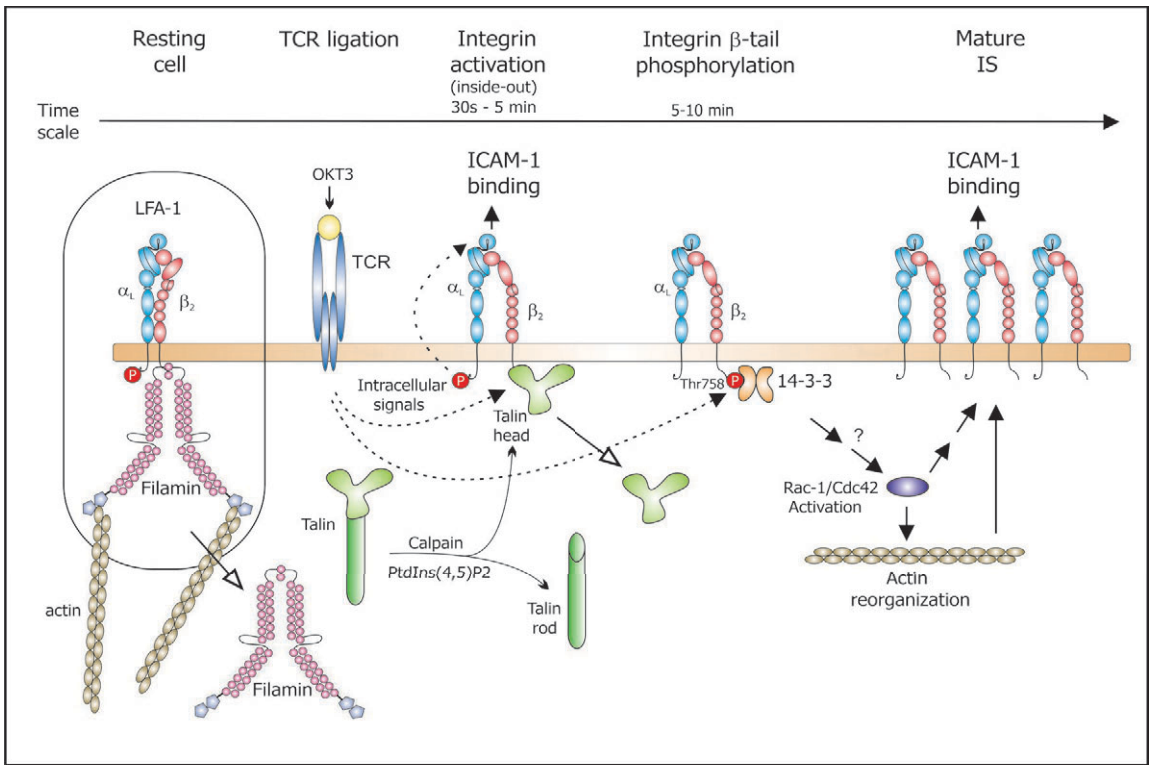

Fig 10. Schematic diagram of intracellular events examined in this thesis.
In resting T cells filamin is bound to the unphosphorylated β2 integrin tail. TCR ligation leads to the activation of several different signaling pathways. At the early stages of T cell activation, talin head and rod domain are separated and the head domain binds to the NPxF motif in the β2 integrin tail, thus inducing a conformational change in the integrin, which results in high-affinity binding to ICAM-1. The phosphorylation of the β2 tail takes place in the later stages of T cell activation and allows the 14-3-3 protein to bind to the Thr758 phosphorylated integrin. This binding results in Rac/Cdc42 activation and ultimately leads to actin reorganization and cell spreading, which further strengthens the cell-cell adhesion.

The phosphorylated integrin-14-3-3 complex then mediates the activation of Rac-1/Cdc42 through some intermediate steps, which still remain to be solved, ultimately leading to actin reorganization, cell spreading and strengthening of the cell-cell contact. At this point the extended high-affinity integrin activated by talin binding has bound ligand, which also sets in motion the LFA-1 outside-in activation. The signaling through the integrin further activates down-stream signaling cascades re-enforcing the actin reorganization and cell spreading, so called post-receptor events. LFA-1 also mediates costimulatory signals to fully activate the T cell and to achieve the mature IS.

CONCLUDING REMARKS

Naïve T lymphocytes continually recirculate by crossing the high endothelial venules into lymph nodes and further on into the lymphatic circulation after which they re-enter the blood stream. If contact is made with the APC, an immune response is initiated. The immune system relies on dynamic adhesion events for proper function and the LFA-1 integrin, which is exclusively expressed in leukocytes, is an important mediator of these adhesive events. LFA-1 mediates adhesion under various conditions, such as during immunological synapse formation and during leukocyte emigration. Whereas chemokine-induced adhesion is fast and rapidly reversible, TCR-mediated adhesion is slow and sustained. Because of this variability of the adhesion events in the immune system, multiple mechanisms for regulation are likely to be employed. Both affinity-dependent and independent mechanisms have been postulated to be important in the regulation of integrin activation. Ligand binding or chemokine triggering are known to mediate rapid conformational changes in LFA-1 leading to high-affinity integrin, however also clustering of integrins is involved after ligand or chemokine triggering. On the other hand, TCR-induced activation of LFA-1 does not involve affinity regulation of the integrin, but instead has been closely connected with actin cytoskeleton rearrangements and a spreading phenotype of T cells. However, none of these mechanisms by which integrin-mediated adhesion is regulated are mutually exclusive and different modes of regulation can and are likely to work in combination.

The results of this thesis demonstrate the importance of phosphorylation of the intracellular tails of LFA-1 integrin in the regulation of integrin function. αL phosphorylation is needed for rapid changes in the conformation and affinity of the integrin heterodimer, whereas β2 phosphorylation on Thr758 after TCR ligation functions as molecular switch to inhibit filamin binding and allow 14-3-3 binding. Talin binding to β2 tail is also indirectly inhibited by Thr758, since 14-3-3 outcompetes talin for binding to the phosphorylated integrin. The 14-3-3-β2 integrin complex also signals downstream of the integrin through intermediate steps to activate the small GTPases Rac1 and Cdc42, which ultimately leads to rearrangement of the actin cytoskeleton and cell spreading. Furthermore, Thr758 phosphorylated β2 integrin peptide was observed to induce downstream signaling (expression of the T cell activation marker CD69) in a similar manner to TCR antibody together with ICAM-1. The down-regulation of Cbl-b was also found to be essential in mediating the activation of LFA-1 through Thr758 phosphorylated β2 integrin and 14-3-3.

Improper function of LFA-1 contributes to a non-functional immune system or autoimmune diseases. Thus, it is of fundamental importance to understand the different mechanisms regulating integrin functions. The results of this thesis reveal novel mechanisms involved in regulating LFA-1 function and hopefully will assist in the pursuit of new and more effective therapeutics for autoimmune diseases.

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